MUCUS SECRETAGOGUE ACTIVITY IN CECAL CONTENTS OF RABBITS WITH EXPERIMENTALLY-INDUCED MUCOID ENTEROPATHY

By

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To Mark, Laura, and Arthur who never let me forget what is really important.

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KEY TO ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
H&E	Hemotoxylin and eosin
HBSS	Hank's balanced salt solution
kDa	Kilodalton
ME	Mucoid enteropathy
NZW	New Zealand White
OPD	O-phenylene diamine
PBS	Phosphate-buffered saline
PBS-T20	Phosphate-buffered saline-tween 20
PMSF	Phenylmethylsulfonyl fluoride
SBA-HRP	Soybean agglutinin-horseradish peroxidase
SEM	Standard error of the mean
SPF	Specific pathogen-free
VIP	Vasoactive intestinal peptide

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Mucoid enteropathy is a disease of rabbits, characterized by copious mucus secretion in the intestinal tract. The etiology of this disease is unknown. This study uses an adaptation of the cecal ligation model developed by Toofanian and Targowski to induce experimental mucoid enteropathy. Filtrates of cecal contents from these rabbits and from control animals were prepared. Explants from healthy rabbits were incubated with these filtrates, and mucus secretion was measured using an enzyme-linked lectin assay, or by measuring secretion of radiolabelled mucus from explants that had been preincubated with ^{3}H-glucosamine. The results demonstrate the presence of a secretagogue for colonic mucus in the cecal contents of rabbits with experimentally-induced mucoid enteropathy. Pretreatment of filtrates demonstrates that this secretagogue can be precipitated with ammonium sulfate, and is

destroyed by heat (100°C for 30 minutes) or strong acid (pH 1 for 30 minutes).

CHAPTER 1
INTRODUCTION

Mucoid enteropathy (ME) is a disease that affects only rabbits. It primarily affects young animals, but it can occur at any age (105). Mortality from mucoid enteropathy has been reported as 1-2% (238) to 10-20% (105, 251) of rabbits kindled. Signs associated with the disease include anorexia, dehydration, depression, subnormal temperature, distended abdomen, and the defecation of clear to yellow gelatinous mucus in the feces (238). Afflicted rabbits may also have diarrhea, or may pass nothing but mucus (208). There is disagreement as to whether or not affected rabbits drink water. In our experience, and in some reports (235), they are adipsic; but many reports describe polydipsia (238, 251). Clinically, the rabbits are dehydrated. Afflicted rabbits may die acutely, or after several days of illness. Affected rabbits may recover, with or without intervention (105).

At necropsy, the consistent finding is copious amounts of clear to yellow gelatinous mucus in the colon. The small intestine may contain fluid and/or mucus. The cecum is often impacted with dry digesta, which may contain gas pockets. The gallbladder is often distended. Histologically, there may be goblet cell hyperplasia, most noticeable in the ileum but may

occur in any part of the gastrointestinal tract. There is minimal histologic evidence of inflammation, with mild mixed infiltrates seen inconsistently (142, 238, 251).

Some reports of "mucoid enteritis" (142, 163) describe gross paintbrush hemorrhages of the large intestine, and microscopic edema and inflammation. Such lesions are more characteristic of clostridial enterotoxemia, a more recently defined disease of young rabbits (20), which is caused by an overgrowth of toxin-producing clostridial organisms, *C. spiroforme* or *C. difficile* most commonly. This disease is usually initiated by antibiotic administration or overeating of a high calorie, low fiber diet. Clinical signs of enterotoxemia include anorexia and dehydration, as in mucoid enteropathy. However, rabbits with enterotoxemia usually have watery diarrhea and tend to die acutely. Interestingly, animals with enterotoxemia can also pass large quantities of mucus, suggesting that mucoid enteropathy may be a syndrome with many possible etiologies, rather than a specific disease.

Studies of the cause and treatment of mucoid enteropathy has thus far been unrewarding. Several studies have concentrated on potential infectious causes, especially since the incidence of the disease occurs in "outbreaks." Greenham (64) found that tetracycline decreased diarrhea and delayed death, but did not prevent or cure the disease. Van Kruiningen (238) created an oral inoculum of macerated intestine and contents in an attempt to transmit the disease.

Only 5 out of 17 of these transfaunated rabbits became ill, while 2 out of 17 rabbits fed a control inoculum also developed mucoid enteropathy. Intestinal coliform concentrations are often increased in animals with mucoid enteropathy (64, 113, 207); however inoculation with *E. coli* obtained from rabbits exhibiting diarrhea, or *E. coli* heat labile enterotoxin (207) failed to induce disease. Lelkes and Chang (114) found several differences between the cecal flora in normal rabbits and in those with mucoid enteropathy, but could not identify a single organism associated with the disease. They also transfaunated cecal contents from sick rabbits directly into the ceca of healthy fistulated rabbits and were unable to cause increased mucus secretion.

Research on ME has generally focused on determining the inciting cause. There could very well be more than one etiology. Individual cases have been seen in adult rabbits with debilitating and/or stressful conditions, such as neoplasia, surgical stress, and septicemia (Hotchkiss, unpublished observations). The present study was therefore designed to focus on the direct cause of the increased mucus secretion, the one consistent finding in all afflicted animals.

In the past, studies of mucoid enteropathy have been hampered by the lack of viable models of the disease. There is now one model available: based on the hypothesis that mucoid enteropathy is primarily due to constipation,

Sinkovics (207) was able to induce colonic mucus hypersecretion by surgical ligation of the colon. Toofanian and Targowski (235) found that ligation of the blind pouch of the cecum, permitting the flow of ingesta from ileum to colon, was just as effective, causing mucus hypersecretion in approximately 70% of rabbits. Partial cecectomy did not induce a mucoid enteropathy-like syndrome, while injection of tetracycline into the ligated cecum prevented the development of disease (235). Little work has been done with the cecal ligation model, however. Toofanian's group found decreased small intestinal disaccharidase activity in ligation-induced sick rabbits (233), and also minor alterations in cecal volatile fatty acid concentrations (234). They incubated colonic explants with cecal contents from rabbits with experimentally-induced mucoid enteropathy and reported goblet cell hyperplasia (236).

In the project described herein, methods have been developed to test this finding in a quantitative manner. Evidence is presented to support the hypothesis that cecal contents from rabbits with mucoid enteropathy possess mucus secretagogue activity. Preliminary tests have been carried out to determine the nature of the secretagogue.

The importance of doing research on this disease is twofold: First, little is known about it, considering that it is one of the most important causes of morbidity and mortality in rabbit production (113). Since rabbits are used in large

numbers in biomedical research, these losses can add up to substantial costs, especially in situations where affected animals have been injected for antibody production with very precious and irreplaceable antigens. Second, this disease provides a unique opportunity to study the control of mucus secretion. Increased mucus secretion without inflammation can be seen in enterotoxic diseases, such as cholera and *E. coli* enterotoxemia, but there is no associated goblet cell hyperplasia (96), and there are other confounding toxic effects, notably a secretory diarrhea. On the other hand, goblet cell hyperplasia with mucus hypersecretion can be seen with some diseases and parasitisms (96), but because of the associated inflammatory changes it is difficult to examine the mucus related aspects alone.

Although the secretagogue effects of specific chemicals such as acetylcholine and PGE_2 are well known (160, 258), their physiological importance is not always clear. This study is intended to demonstrate the presence of a secretagogue that is pathologically relevant, with few effects other than the stimulation of mucus production. It may be a microbial product or a host secretion; in either case it could prove to be useful for studying the processes controlling differentiation of goblet cells, as well as production and secretion of intestinal mucus.

CHAPTER 2
LITERATURE REVIEW

Introduction

In studying a disease in which the most consistent sign is mucus hypersecretion, it is necessary to examine the role of mucus in the normal physiological state. This review presents information on what mucus is, what controls its synthesis, secretion, and degradation, and what the normal functions of gastrointestinal mucus are.

The function of mucus has been a subject of study since ancient times. The ancient Greeks considered mucus, commonly translated as "phlegm", to be one of the four humors, along with blood, yellow bile, and black bile. In the second century Galen (56) stated, "The best physicians concur in the opinion that if a considerable amount of phlegm accumulates on account of some bad conditions of the body, the most serious abdominal disorders ensue, intestinal obstruction, lientery, and tenesmus". Although it is now generally believed that mucus is involved in lubrication rather than obstruction, there is no question that increased mucus production occurs in certain disease states.

Characteristics of Mucus

Physical Characteristics

Initially, the term mucus was used to describe any viscous material in the stomach and intestines. Primarily, mucus was the jelly-like material which lined the gastrointestinal tract. However, for a time, the material which could be precipitated from gastric juice with suitable agents was known as "dissolved mucin" (9). However, as it was found that this material contained a heterogenous mixture of substances, this terminology was dropped (59). Still, mucus was variably described as clear and colorless, white and cloudy, and occasionally yellow. Hollander *et al.* (81) determined that these differences were due to the amount of cells and debris dissolved in the mucus layer. They were able to show that the irritants used to stimulate mucus secretion resulted in desquamation of epithelial cells, associated with inflammation (80).

Pilocarpine injected intravenously causes increased secretion of mucus of egg-white consistency; however, an enormous dose is required (44). In 1949, Morton and Stavraky (153) found that injection of acetylcholine into the mesenteric arteries caused secretion of mucus, while Janowitz *et al.* (92) found that topical administration of acetylcholine resulted in secretion of clear, cell-free mucus, facilitating the study of "pure" mucus. It is now generally accepted

terminology that mucin is a specific glycoprotein, while mucus is a heterogenous mixture containing mucin, protein, lipids, nucleic acids, water, and electrolytes. Specifically, mucus contains trefoil-type protein, kallikrein protease, lactose-binding lectins, vitamin B_{12} binding protein, and other proteins which are secreted with mucin from the same granules in the goblet cell (178). In the respiratory tract, most of the lipid in the mucus is bound to the mucin glycoprotein (157). Also, nucleic acids entwine other proteins with mucin, making acidification and nuclease treatment necessary in the purification of mucin (157). In the digestive tract, the mucus layer also includes bacteria, food particles, and products of digestion. In disease states, such as cystic fibrosis, mucus also contains materials from degenerated leukocytes, especially DNA and actin which contribute to its viscosity, and interfere with clearance (239).

The most striking feature of mucus is its jelly-like nature. Early studies revealed that unlike most proteins, which show minimum viscosity at the isoelectric point, gastric mucin shows a maximum viscosity at its isoelectric point of 4.98 (144). As rheological studies have become more sophisticated, it has been shown that mucus is a visco-elastic gel; and that the elastic component is greater than the viscous component over the entire range of frequency and strain studies with native and reconstituted mucus (4). The elastic properties will be decreased, allowing the gel to

collapse if the mucin is treated with proteolytic enzymes or if disulfide bonds are reduced with mercaptoethanol (4). In addition, proper hydration is necessary for maintenance of the gel. Hydration is controlled by small polyionic proteins, pH, and electrolytes within the gel (242). Since mucin is a negatively charged glycoprotein, a high concentration of a positively-charged ion such as calcium can result in condensation of the mucus gel, with an increase in viscosity and lack of elasticity.

The chemical properties of mucin have been slowly elucidated over the past century. Mucins are a heterogeneous group of glycoproteins, with an average subunit molecular weight of 2×10^6 (220). Carbohydrate comprises approximately 80% of this molecular weight, with the rest being protein. The protein forms a core, with linear and branched carbohydrate side chains covalently attached to form a "bottle brush" structure (126). As well as heavily glycosylated areas, there are believed to be "naked" areas on the protein core, which are subject to cleavage by proteolytic enzymes (4). These regions also contain cysteine residues, allowing for polymerization via disulfide bonds (160), and are the major antigenic determinants of mucin (131). The glycosylated portion of the protein core contains a high proportion of serine and threonine, which are involved in oligosaccharide linkage via hydroxyl groups (220). In addition, there is a

high percentage of proline, which prevents α-helix formation, allowing for increased glycosylation and flexibility.

The oligosaccharide chains of mucin are attached to the protein core via an O-glycosidic bond between serine or threonine and N-acetylgalactosamine (220). These chains may be linear or branched, and vary in length from 2 to 12 residues. Some chains will terminate in A, B, H, Lewis^{a}, or Lewis^{b} antigens, reflecting the blood group genes of the individual (41). The backbone of each chain contains N-acetylgalactosamine, N-acetylglucosamine, and galactose, which may comprise I or i antigens (126). The chains often terminate in a fucose, sialic acid, or ester sulfate residue. The glycosylation pattern depends on genetics, the region of the gastrointestinal tract, the age of the animal, and can vary in certain disease states, as determined by the glycosyltransferase activity levels (228). There is also a great deal of heterogeneity in a single preparation, even within a single granule of a goblet cell (220).

The DNA sequences of several mucins have been determined (116). These genes show a high degree of polymorphism due to variable numbers of tandem repeats. The gene MUC1 encodes a mucin-like cell surface glycoprotein which is present on many epithelial cells, but MUC2 to MUC6 appear to encode secreted mucins. MUC2 and MUC4 are associated with colonic mucins, and MUC3 and MUC5 are associated with gastric mucins (116).

For several years it was debated whether or not mucin contains a "link" glycopeptide, of approximately 118 kDa (160). Roberton *et al.* (188) demonstrated that this glycoprotein is different from other mucin subunits, in that it contains only 50% carbohydrate, and it contains mannose, which is generally associated with N-linked glycosylation (220). Amino acid composition, carbohydrate composition, and immunological studies have indicated that the "link" glycopeptide is actually a 118 kDa fragment of fibronectin (210). However, the cDNA for this peptide has recently been sequenced in rats (257) and humans (256), and the 118 kDa glycopeptide appears to represent the cysteine-rich carboxyl terminal of a much larger mucin-like peptide. It is now generally accepted that proteolytic cleavage of the mucin core peptide occurs during purification, and that reduction and alkylation allows for separation of the larger N-terminal fragment and the 118 kDa C-terminal fragment (262).

In this chapter, there has been some generalization in order to describe the overall functions of mucus. However, it is important to recognize that mucin molecules are heterogeneous, and some of the characteristics of mucus vary along the length of the gastrointestinal tract, change during development and maturation, and can be altered by disease (160). In fact, a diurnal rhythm to mucus secretion has been seen in rats and mice which were given access to food only in

the daytime: there was more cell proliferation, and more mucus was made during the daytime (73).

The mucin produced in newborn rats is different from that found in adults (160). Newborn mucin contains more protein and less sugar, resulting in increased density (216). The threonine content is increased, while serine and glutamic acid content are decreased in newborn rats (160). Sulfation decreases in the first 2 months of a rats life, with the major change occurring at weaning; increased sulfation has also been associated with mucus from "immature" cells (160). Correspondingly, the pH within the mucus layer in the colon is lower in suckling rats than in weanling or adult rats (193). Aged (350 day old) rats also have increased sulfation of mucus (225).

Newborn rat mucin has fewer fucose and N-acetylgalactosamine residues, corresponding to altered lectin binding (216). This likely relates to developmental changes in glycosyl-transferase activities (12). During the suckling period, N-acetylgalactosaminyl-transferase activity remains constant, sialyl-transferase activity decreases just prior to weaning, while fucosyl-transferase activity decreases gradually throughout the suckling period. Following weaning, the activities of all three enzymes increase dramatically. In a parallel manner, more sialyl-transferase can be detected immunocytochemically in the mucus of mature goblet cells, when compared to immature goblet cells in the colonic crypt (226).

Sialylation of surface glycoproteins is replaced by fucosylation at weaning, but sialylation of goblet cell mucus continues (227).

The composition and structure of mucin varies along the length of the gastrointestinal tract. The data on the actual differences are sometimes contradictory, due to different techniques, species differences, and the heterogeneous nature of all mucins (160). In general, colonic mucin is more aggregated, and contains more protein. It may not contain the fragment that was formerly known as the "link" glycopeptide. The concentration of acidic glycoproteins, containing sialic acid and/or sulfate, relative to neutral glycoprotein increases distally in the digestive tract. The gastric and Brunner's gland secretions are largely neutral, lacking sialic acid, but enriched in fucose, although in gastric mucin there is a discrete subpopulation of highly sulfated mucin associated with the mucous neck cells (90).

The viscosity of gastric mucus is greater than that of duodenal mucus, while the elasticity is decreased (263). In the stomach, there is a continuous layer of mucus, but the thickness is variable, and tends to be thickest along the lesser curvature (165). Mucus is present in the small intestine, but mostly in the intervillous spaces, so that the tips of the villi are sometimes visible. There is less mucus in the ileum than in the duodenum and jejunum, so that most of the villi are bare *in vivo* (165), although a layer 50-200μ

thick can be seen in explants (67). There is no distinct mucus layer in the cecum (165, 194). In the proximal colon there is a discontinuous layer of mucus, varying in composition and thickness, while in the distal colon there is a thin, compact, continuous mucus layer present (194, 225). Mucus from the healthy colon is more hydrophobic than ileal mucus; however, induced colonic inflammation decreases the hydrophobicity (124).

Smectite is a cytoprotective agent that binds to mucus to increase its barrier properties. It also causes gastric mucus cells to produce mucus that contains more fucose and less sulfate, as is seen with fasting (151). Dietary fiber has been shown to increase the rate of turnover of intestinal mucins, and also change their lectin-binding properties, reflecting a change in composition (152). This is consistent with the fact that alterations in glycosyltransferase activities are seen with different diets (12).

Mucus can be abnormal in various disease states. Although in cystic fibrosis the major mucus abnormalities are likely decreased hydration (160, 242) and the presence of degenerated leukocyte components (239), some investigators have also found an increased fucose content (231). Celiac disease (gluten-related enteropathy) results in a decreased number of goblet cells, and a shift from neutral to acidic mucin (160). Atrophic gastritis is associated with decreased epithelial mucus, either due to decreased secretion or

increased degradation (135). In mucoid enteropathy of rabbits there are increased numbers of immature colonic mucins with heterogeneous lectin binding patterns, perhaps due to the increased rate of turnover (89). Abnormal mucins are also seen in infection of rats with *Nippostrongylus brasiliensis* (88).

Goblet cell numbers are markedly reduced in patients with ulcerative colitis (160). In addition, there is a change in the composition, with decreased fucosylation and sulfation, and exposure of galactose residues. This, along with deficient O-acetylation of sialic acid (94), is suggestive of incomplete mucin glycosylation. Intestinal malignancy is also often associated with loss of terminal sugars, decreased O-acylation of sialic acid, and shortened oligosaccharide chains (160). Consequently, the presence of small intestinal mucin antigen (SIMA) in the colon, where there is normally masking of antigens by sulfate, may reflect malignancy (43). SIMA can be detected in the serum of 36% of colorectal cancer patients vs. 5% of controls, and may be useful as a screening test (179). The abnormal glycosylation appears to be a clonal phenomenon, reflective of somatic mutation (55). Gastric adenocarcinomas are abnormally rich in sulfomucin and sialic acid, which may reflect cellular dedifferentiation, or abnormalities in the regulation of mucin biosynthesis (160).

Production

Mucus is formed by several cells in the gastrointestinal tract. In the stomach, mucus is secreted by surface cells, the mucous neck cells of the fundus, and the mucous cells of the cardiac and pyloric glands. In the duodenum, the mucus-secreting cells are the Brunner's glands. Throughout the rest of the intestine the goblet cells are the source of mucus (47). Goblet cells are named for their characteristic goblet shape seen in histologic section, but electron microscopic studies on cryofixed tissues show that these cells are columnar *in vivo* (84, 242).

Duthie (38) reported that the mucus granules are first formed near the nucleus, then are transported to the Golgi apparatus, where stainable mucus first appears. This is consistent with current knowledge that the peptide backbone, which comprises less than 50% of each mucin molecule, is formed in the endoplasmic reticulum. Sugar residues are then added by the linkage of N-acetylgalactosamine to serine or threonine (19). Unlike N-linked glycosylation that is initiated in the endoplasmic reticulum, O-linked glycosylation occurs entirely within the Golgi and condensing vacuoles (63). The initial enzyme involved, α1,3N-acetylgalactosaminyl-transferase, is located in the Golgi apparatus (191). The enzyme responsible for terminal sialylation, ß-galactoside α2,6-sialyltransferase, is present in the Golgi apparatus, but also in post-Golgi apparatus structures, including the mucus

droplets and the plasma membrane (228). The mucin granules are stored in condensed form in membrane bound vesicles within the goblet cells until the mucus is secreted. During goblet cell migration along the middle half of the villus, the mucin granules are apparently renewed twice (23). The composition of mucin in the granules is different along the crypt-villus axis (23, 160); perhaps there is continued sialylation of mucin within the storage granules.

The rate of mucin synthesis depends on the rate of protein synthesis within the cell, the concentration and positioning within the cell of glycosyltransferases, and the presence of amino acid and sugar precursors (160). Glutamine is known to stimulate glycoprotein synthesis as the major metabolic substrate in the intestinal epithelium (51), but little is known about the control of internal cellular factors.

Mucus synthesis can be decreased by factors which interfere with protein or oligosaccharide incorporation. Factors which interfere with protein synthesis will also decrease mucus production. These include malnutrition, and metabolic inhibitors such as cycloheximide and puromycin (160). Dietary fatty acids, non-steroidal antiinflammatory drugs, and zinc diminish sulfation or interfere with incorporation of individual sugars (51, 160) and may thereby alter the composition of mucus. Cysteamine, which is used to

experimentally induce duodenal ulcers, decreases glycoprotein production in Brunner's glands (104).

ß-Adrenergic drugs, cyclic AMP, and theophylline have been shown to increase overall glycoprotein synthesis in rat and rabbit intestine (50, 110). Adrenergic compounds (dopamine, epinephrine, isoproterenol, phenylephrine) have no direct effect on mucus secretion, although they may increase fluid secretion, making the mucus blanket appear thicker (160). Cholinergic drugs and cholera toxin increase both synthesis and secretion of mucus (198). Reserpine, a hypotensive agent and carcinogen, increases glycoprotein synthesis; the mucin that is released is more viscous than normal, so that reserpine-treated rats have been used as a model for cystic fibrosis (169). Epidermal growth factor has been shown to increase glycosaminoglycan synthesis, and has a protective effect on the gastric mucosa, but its relationship to mucus secretion has not been studied (209).

Histamine increases mucin synthesis in the canine stomach via H_2 receptors, through activation of adenylate cyclase and increases in cAMP (198). Gastrin increases mucus synthesis in the corpus of the rat stomach, and this effect is not blocked by H_2 blockers (86). Conversely, the H_2 blockers roxatidine and FRG-8813, but not cimetidine or ranitidine, increase mucin synthesis (87). Additionally, the H^+,K^+-ATPase inhibitor NC-1300-O-3, but not omeprazole, stimulates mucin synthesis (87).

Anatomic alterations which affect the number of goblet cells present and/or the rate of development will also change the amount of mucus synthesized. Duodenal epithelial cells from chick embryos show an increased number of goblet cells when cultured *in vitro* compared to the number seen *in vivo* (13). This increase in goblet cells is accelerated by thyroxine, but prevented by hydrocortisone. Vitamin A deficiency causes atrophy of goblet cells in salivary glands, trachea, and small intestine (30). *Salmonella* infection in mice decreases the number of goblet cells, apparently via tumor necrosis factor α (8). Feeding of dietary fiber has been associated with increased turnover of jejunal mucins (152). Some substances which damage the intestinal epithelium, such as methotrexate, will decrease the total number of goblet cells, and therefore decrease mucin synthesis (95). Following radiation damage (154), or the radiomimetic disease caused by canine or feline parvovirus infection (96), there is an initial increase in the number of immature goblet cells, followed by a relative decrease, and then a second increase as the tissue recovers. Corresponding changes in mucus production take place following radiation exposure (201). A decreased proportion of goblet cells is seen in lesions of transmissible colonic murine hyperplasia caused by *Citrobacter freundii* biotype 4280, and is followed by goblet cell hyperplasia during recovery (10).

Several organisms have been associated with goblet cell hyperplasia leading to mucus hypersecretion, including *Treponema hyodysenteriae* in swine, and *Ostertagia and Oesophagostomum* species in ruminants (36, 96). These organisms are also associated with a varying degree of inflammation, so the hyperplasia may be a response to inflammation, repair, or the organism.

Nippostrongylus braziliensis infection in rats causes goblet cell hyperplasia as the worms are being expelled (147). This appears to be associated with the immune response, since hyperplasia will be seen earlier in immunized rats, and does not occur if the rats are treated with antihelmintics early in the course of infection. *Trichinella spiralis*, *Nematodirus battus*, *Trichostrongylus tenuis*, and *Hymenolepis dimunuta* have all been associated with goblet cell hyperplasia (111, 139).

Yersinia enterocolitica has been shown to cause goblet cell hyperplasia and increased mucin synthesis throughout the intestinal tract of rabbits (129). There is also a great deal of inflammation associated with this disease. The goblet cell hyperplasia develops more rapidly and to a greater extent in those areas of the intestine where mucosal injury is most severe, and consequently may be associated with injury and or inflammation. Alternatively, the hyperplasia may be part of a repair mechanism, since it persists as the mucosa recovers morphologically.

Intestinal coccidiosis caused by *Eimeria* species has been associated with inflammation and goblet cell hyperplasia in rabbits (114). Mucoid enteropathy, the subject of this study, is characterized by copious production of intestinal mucus, where the major histologic change reported is goblet cell hyperplasia, and inflammation is minimal (238).

On the other hand, it is important to realize that goblet cell hyperplasia in response to infection and/or inflammation is not a universal phenomenon. Mucus secretion is decreased in both small and large intestines during infection with *Isospora suis* in piglets (106, 107), and numbers of colonic goblet cells are decreased with *Ehrlichia risticii* in horses (187). *Clostridium difficile* toxin A had no effect on goblet cells in rabbits (118).

Secretion

Mucus is secreted from intestinal goblet cells in two ways. "Baseline", or constitutive, secretion, involving the slow transport and secretion of glycoproteins that can be documented by autoradiography, occurs in mucosal explants for up to 24 hours in the absence of circulating factors or enteric nerves. In fact, baseline secretion occurs even in a cultured goblet cell line (173). While the bulk of the mucin granules remain in the center of the goblet cell after formation, there is constant formation of mucus at the supranuclear region of the cell. This mucin migrates along

the periphery towards the apical border of the cell over the course of 4 to 6 hours (184). Thus, some newly formed mucin reaches the apical border and is secreted before older mucin granules (23). A single granule will undergo exocytosis, discharging mucus into the lumen. This process apparently involves the cytoskeleton, as depolymerization of microtubules with colchicine prevents this migration (219). However, once the granule has reached the apical membrane, the actin filaments localized there normally act as a barrier, since depolymerization of actin filaments with cytochalasin D or dihydrocytochalasin B results in increased baseline secretion (167). Organelles are gradually shed from goblet cells as mucin is secreted, so that the mean cell volume decreases along the crypt-villus axis (184).

In the cases where it has been examined, stimulated secretion has been shown to occur by compound exocytosis (217). In this situation fusion of the initial mucin granule with the plasma membrane is rapidly followed by tandem fusion with the subjacent granules, allowing the contents of many granules to exit through a single surface site. More recent studies suggest that there is also fusion of granules within the cell prior to exocytosis (84, 158). Cavitation in a goblet cell that has just discharged its granules in this manner can be recognized by electron, or even light, microscopy (217). Following cholinergic stimulation, cavitation is visible in crypt cells for 15-30 minutes; after

that it is difficult to distinguish mucin-depleted goblet cells from epithelial cells (178). Cavitation may never be apparent in villous goblet cells (178). In rabbit colonic goblet cells stimulated by acetylcholine, compound exocytosis is not inhibited by colchicine, implying that microtubules are not necessary, since the granules are already at the site of release (219). However, the protein synthesis inhibitor cycloheximide, the microtubule inhibitor colchicine, and the actin inhibitor cytochalasin B were all found to inhibit cholera toxin stimulated secretion in rabbit ileal loops (164). A jack-in-the-box mechanism for secretion has been proposed (242), in which a small pore is opened over the granule, water is allowed to enter and calcium can leave, with the expansion of the granule contents causing release from the cell.

It has been reported recently that mucus secretion is stimulated independently by cAMP/protein kinase A and increased intracellular calcium/protein kinase C mechanisms (93), although previous reports state that cAMP does not affect mucus secretion (161). *In situ* hybridization reveals the calcium binding protein calcyclin preferentially expressed in mucus-secreting cells, suggesting that calcyclin, in conjunction with the p36 subunit of calpactin, is involved in calcium-stimulated mucus secretion (232).

Several substances are known to stimulate mucus secretion. Agents that disrupt the mucosal barrier, such as

mustard oil, alcohol (160), bile salts (117), and even mechanical irritation (51) cause mucus release from surface goblet cells. It has been shown that mustard oil causes compound exocytosis (217). Florey (45) demonstrated that secretion in response to mustard oil could be blocked with cyanide, indicating that the process requires energy, and secretion is not simply due to disruption of the plasma membrane. Mechanical irritation has been shown to increase levels of prostaglandins (26), which could be acting as secondary messengers to stimulate secretion. Crypt goblet cells are not affected, presumably because they do not come into contact with the irritant. Proteolytic enzymes are known to be mucus secretagogues in respiratory epithelium, but this has not been shown in the intestine (22).

Stimulation of extrinsic autonomic nerves or electrical field stimulation causes discharge of intestinal mucus *in vivo* (175). This has been shown to be due to muscarinic cholinergic innervation, as injection of pilocarpine accelerates release of mucus by compound exocytosis. Vagotomy or vagal stimulation does not affect mucus secretion in the rabbit jejunum, implying that cholinergic control takes place entirely within the enteric nervous system (65). Acetylcholine-induced secretory events occur rapidly, and are generally complete within 5 minutes (158, 178). It was long thought that only crypt cells were susceptible to stimulation (218), but it is now known that villous cells secrete a

significant amount of mucus (100), although they do not demonstrate cavitation (178). In the rat, even crypt cells are unresponsive to carbachol until 20 to 25 days of age, corresponding to weaning (176). Acetylcholine (218), pilocarpine (219), and carbachol (174) have also been shown to stimulate mucus release in colonic explant systems. Furthermore, it is fairly certain that the goblet cells themselves possess muscarinic receptors, as secretion occurs in a goblet cell line descended from a colonic adenocarcinoma (173). The response is variable, but this may reflect the variation of responsiveness *in vivo* between crypt cells and the older surface or villous cells.

Prostaglandins, particularly PGE_2, increase mucus secretion, and perhaps synthesis (197, 198, 258). Non-steroidal antiinflammatory drugs and indomethacin decrease mucus synthesis and secretion, and it has been proposed that inhibition of prostaglandin synthesis may be responsible (164, 206). Glucocorticoids also decrease mucus secretion (141). Low doses of nicotine greatly decrease rectal prostaglandin levels and decrease the thickness of the mucus layer, while high doses cause an increase in mucus thickness and decrease prostaglandin concentrations less dramatically (266). Other arachidonic acid metabolites (leukotrienes, hydroxyeicosatetranoic acids) act as mucus secretagogues in respiratory epithelium (171), but do not affect rabbit colon *in vitro* (177).

Gastrointestinal peptide hormones have also been suggested as potential mucus secretagogues. Neutra *et al.* (161) found no increase in compound exocytosis in rabbit intestinal explants when stimulated with caerulein, cholecystokinin, pentagastrin, secretin, somatostatin, substance P, or vasoactive intestinal peptide (VIP). However, some studies indicate that secretin increases mucus secretion in the stomach (99), and that both secretin and the related hormone VIP weakly stimulate colonic mucus secretion *in vivo* (40). Recently, VIP receptors have been found on mucus-secreting cells in culture (108). VIP alone does not stimulate mucus secretion from these goblet-like cells, but both VIP and cAMP potentiate the secretagogue effects of carbachol. This potentiating effect of VIP has also been seen in tracheal submucosal glands (204).

The vasoactive amine histamine causes increased colonic mucus secretion (161), but only under nonphysiologic culture conditions (160). Histamine has also been shown to increase PGE_2 levels, and so may act indirectly (247). A greater amount of gastric mucus was recovered after stimulation with serotonin (141); however, there was no morphologic evidence of increased secretion in rabbit colon following serotonin stimulation (160).

Several inflammatory products of neutrophils, macrophages, and mast cells have been shown to stimulate mucus secretion from respiratory epithelium (70, 160), but they have

not been studied extensively in the intestine. There is now increasing evidence that interleukin 1 (IL-1) increases intestinal mucus release (27, 69). In addition, a macrophage product, MMS-68, first isolated from the respiratory tract, has been shown to stimulate intestinal mucus secretion (221). Immune complexes have been shown to induce compound exocytosis of mucin (246). Antigen challenge following oral, but not intraperitoneal, inoculation increases mucus secretion, suggesting involvement of mucosal immunity (109). When a jejunal self-filling blind loop is created, causing bacterial overgrowth, there is increased mucus secretion within the loop, but it is decreased outside the loop (202). This phenomenon could be the direct result of the organisms, or a secondary response to inflammation.

Cryptosporidium parvum infection in mice resulted in an increased amount of mucus in ileal washings (77). It is not clear whether more mucus was actually secreted or if it was simply dislodged more easily. If secretion was increased, it is still not possible to tell if the effect is a direct result of the organism, or secondary to some inflammatory mediator.

Virulent strains of *Entamoeba histolytica* have been shown to increase secretion of preformed and newly synthesized mucus glycoproteins, and also to increase mucin synthesis in rats, in the absence of an inflammatory response (22). Intestinal trematodes in dogs and cats have been associated with mucoid

inflammatory response, but no quantitative studies of mucus secretion have been performed (96).

Cholera toxin and the heat labile toxin (LT) from *Escherichia coli* both stimulate small intestinal mucus secretion (160). This phenomenon can be separated from fluid and electrolyte secretory effects, which are stimulated by cyclic AMP (189), and blocked by tetrodotoxin (149). The mechanisms for both increased fluid and mucus secretion are complex. Lencer *et al.* (115) have shown that the B subunit of cholera toxin binds to cloned human goblet cells in monolayer culture, but there was no stimulation of mucus release, suggesting that an indirect mechanism may be involved. It is currently believed that binding of cholera toxin to enterochromaffin cells results in increased intracellular cAMP and secretion of serotonin, which in turn stimulates cholinergic neurons (149). Both the fluid and mucus secretory activities of cholera toxin are blocked by capsaicin, supporting the involvement of the enteric nervous system (149). However, fluid secretion is blocked by tetrodotoxin, while mucus secretion is not, suggesting that mucus secretion may be mediated by local effectors released by sensory neurons. In addition, a portion of the capsaicin-sensitive response is also atropine-sensitive, suggesting that there may be a tetrodotoxin-insensitive interaction between cholinergic and sensory nerve terminals occurring in the small intestine.

Although neither cyclic AMP or cyclic GMP alone affects mucus secretion in explant systems (161), Jarry *et al.* (93) found that cAMP directly stimulated both MUC2 gene expression and mucus secretion from goblet cells in culture, in a protein kinase A-dependent manner, which could account for the effects of cholera toxin. This toxin has also been shown to increase mucosal prostaglandin synthesis (183), and the effects of PGE_1 and cholera toxin are qualitatively the same in *in vivo* rabbit intestinal loops (164).

Mucus secretion has been shown to be increased in rats with streptozotocin-induced diabetes, with less mucin present in the tissue than in the luminal washings (134). Synthesis of mucins relative to other glycoproteins is also increased. Interestingly, the intestinal goblet cells from these diabetic rats are no longer responsive to bethanechol or cholera toxin. This phenomenon has not yet been confirmed, and may be an artifact of collection, since fluid secretion is also increased in diabetes, and may flush out the mucus present.

The antiulcer drugs zolimidine, carbenoxolone, quercetin, and oral copper compounds have been reported to increase mucus secretion, but the mechanisms are unknown (2, 3, 51). Parathyroid hormone has been reported to increase gastric mucus secretion (141). The carbonic anhydrase inhibitor, acetazolamide, also is a mucin secretagogue (28). A new antiinflammatory drug, SCH12223, which has protective effects on the gastric epithelium increases gastric mucus content

(25). The amount of gastric mucus that can be aspirated *in vivo* is increased after a meal (98). Feeding increases intestinal mucus secretion, even when the duodenum has been transplanted to a subcutaneous location and separated from its nerve supply (47). Mucus secretion is increased during the daytime in rats that are allowed food only during the daylight hours (73).

The synthetic opioid anti-diarrheal, loperamide, has been shown to slightly decrease baseline mucus secretion, and drastically reduce secretion stimulated by PGE_2 or deoxycholic acid in the rat colon (121). This may be related to neural effects or calcium channel blocking activity (186). Cysteamine, which is used to induce duodenal ulcers experimentally, also decreases mucus secretion (104). Interferon-γ does not affect baseline mucin synthesis or secretion, but inhibits secretion stimulated by both cAMP and calcium ionophores, apparently at the exocytotic step (93).

Degradation

Little mucus is excreted in the feces. It was found that crude mucus undergoes a spontaneous loss of viscosity when incubated overnight at body temperature (72, 91). This phenomenon was accelerated by the addition of certain proteases; different investigators described different sensitivities. It has been shown that pepsin, but not HCl, can dissolve the gastric mucus layer, and cause epithelial

damage (126). That some degradation occurs *in vivo* is supported by the fact that secreted mucin has a lower molecular weight than stored mucin (220).

Although proteolytic enzymes can degrade mucin into smaller glycosylated fragments, resulting in collapse of the mucus gel, and loss of elasticity (4), they cannot completely digest mucopolysaccharides (126). Both α- and ß-glycosidases are required for the removal of sugar residues from mucin (126), and it has been found in humans that these enzymes are primarily derived from *Bifidobacterium* and *Ruminococcus* species (82) in the colon.

Gastrointestinal mucin digestion is markedly decreased in germ-free rodents, due to a lack of glycosidases capable of removing the oligosaccharide sidechains from the peptide backbone (120, 126). This results in an increased mass of mucus in the cecum, increased mucus excretion in the feces, and retention of mucus within a thickened cecal wall. When normal enteric bacterial flora are administered to germ-free rats, excretion of glycoproteins increases for 2-3 days, then drops to the level of conventional animals, confirming that bacterial enzymes play a major role in mucin digestion (120).

As mentioned previously, pepsin partially degrades gastric mucin, resulting in solubilization, and dissolution of the mucus layer. It has been found that patients with peptic

ulcer disease have a higher ratio of pepsin 1 to pepsin 3 than normal individuals, and pepsin 1 is much more efficient at dissolving mucus. Consequently, ulceration may be secondary to the breakdown of the protective mucus (126).

It has been demonstrated *in vivo* that *Helicobacter pylori* infection decreases the thickness of the gastric mucus layer in humans (166, 196). This has been related to the production of protease, lipase, phospholipase, and glycosulfatase by the organisms which impair the protective mucus layer, and may promote mucosal injury. Sulglycotide, a modified sulfomucin gastroprotective agent, acts by inhibition of these enzymes, along with aggregation of the organisms (156, 180). In addition, *Helicobacter* produces urease, and the high levels of ammonia and bicarbonate produced may impair the protection afforded by mucus (35).

Vibrio cholerae contains a virulence factor which consists of a "mucinase complex" (168). Since mucus can bind and inhibit cholera toxin, the destruction of mucus by a metalloproteinase allows the toxin to retain activity, as well as permitting better access to the epithelial cells (32). In addition, a neuraminidase may increase the amount of G_{M1} ganglioside available in the membranes for toxin binding.

Postulated Functions of Mucus

Lubrication

In 1800, Glover postulated that the mucus secretion of the intestinal tract was involved in lubrication (79). Florey (46) saw that particles were removed from the intestine by entrapment in mucus, which was then pushed downstream by the intestinal villi. Nondigestible solids that are emptied from the stomach are entrapped in mucus plugs (66). Mucus has been experimentally shown to aid in ciliary propulsion of objects in tubes (255). The need for lubrication varies with the segment of the intestinal tract discussed. There is little mucus secretion in the small intestine where the contents are fluid, and there is little need for lubrication (47). Conversely, there are many goblet cells within the colon where lubrication is clearly needed for the passage of solid feces (47). More recent studies have shown an incomplete mucus layer in the proximal colon, where the contents still have a high water content, but a thick, compact layer in the distal colon, in order to facilitate the passage of feces (194, 225).

Cytoprotection

In the same thesis, Glover proposed that mucus "must likewise defend the internal surface of the stomach and intestines, from the action of the gastric juice, and from the acritude of bile when regurgitated" (79). Several theories

were put forth for the mechanism of protection. The simplest was that of a diffusion barrier, which will be discussed in a later section. It was believed for a period of time that mucus had direct acid-neutralizing capacity (79). This was first discussed by Pavlov, and studied in detail by Hollander (79). He found a definite buffering capacity in the mucous secretions from Heidenhain pouches of dogs. However, it was shown conclusively by Heatley that mucin itself has minimal buffering power against hydrochloric acid; rather the buffering activity is due to the content of bicarbonate (47). On the other hand, the mucus is responsible for keeping that bicarbonate next to the mucosal surface, as will be discussed later in the section on creation of a microenvironment.

In 1855, Claude Bernard postulated that mucus has a specific chemical or adsorbent action (79). At the beginning of the twentieth century, the presence of a specific antipepsin in mucus was reported (79). However, since 1914 there has been no further evidence of any enzymatic activity of mucus. Bucher (17) showed adsorption of pepsin by the mucus, and Zaus and Foskick (264) and Bradley and Hodges (15, 16) also documented antipeptic effects, however Heatley (74) found that purified mucin does not directly inhibit peptic digestion.

Hydrogen peroxide is rapidly degraded in porcine gastric mucus *in vitro* (34). Although purified mucin is subject to attack by reactive oxygen intermediates, it has been shown

that the lipids associated with native mucus have a protective effect against these radicals (62). Consequently, one function of mucus may be to protect the mucosa from attack by reactive oxygen intermediates released by the host when killing bacteria or as a response to toxins. However, mucus does not affect the cytotoxic activity of *Clostridium difficile* toxin A in rabbits (118).

It has been shown that mucus plays a major role in "adaptive cytoprotection" (21). Following a mild epithelial injury with oleic acid, the thickness of the mucus layer is increased. This prevents injury from a second exposure by delaying the passage of the irritant through the mucus to the epithelial cells. Increased mucus secretion stimulated by the antiulcer drugs carbenoxolone and quercetin protects gastric epithelium from damage by ethanol, 0.6N HCl, or 30% NaCl (2, 3). When acidified ethanol is used for challenge to negate antacid effects, the H_2-blocker FRG-8813 still offers significant cytoprotection through increased mucus production and secretion (85).

Protection from Infection

Cramer (30) found that vitamin A deficient rats are more susceptible to bacterial infections, and related this to the decreased production of intestinal mucosubstance, secondary to decreased numbers of goblet cells. This suggests that mucus can act as a barrier to bacteria. Goldsworthy and Florey (61)

then demonstrated that intestinal mucus contains lysozyme, a non-specific antibacterial enzyme. Specific immunity in the intestinal tract is dependent on secretory IgA. sIgA alone does not prevent bacterial interaction with intestinal epithelium (245), but rather appears to bind to mucin glycoproteins through hydrogen or disulfide bonds (140), allowing for aggregation and neutralization of bacteria.

Because mucus comprises a physical barrier, bacteria have developed mechanisms to allow association with the underlying epithelium. One of these mechanisms is motility. Flagella allow motility of bacteria in aqueous solutions, but are ineffective in viscous environments. However, *Campylobacter jejuni*, although flagellated, acts in high viscosity solution like a spirochete, relying on endocellular organelles for locomotion, and shows an increase in motility related to increased viscosity (42). *Treponema hyodysenteriae*, is similarly highly motile in intestinal mucus (101). This may provide a selective advantage to helical bacteria, such as *Campylobacter*, *Treponema*, *Vibrio*, and *Helicobacter* species, in penetrating mucus and colonizing the intestinal epithelium.

A lipopolysaccharide-deficient mutant of *Salmonella typhimurium* is able to colonize mouse large intestine when given alone, in combination with *E. coli*, or with low concentrations of wild type *Salmonella* (162). However, if high dose of wild type *Salmonella* is given concurrently, or if both strains are allowed to multiply within the host for 8

days, the lipopolysaccharide-deficient mutant is eliminated. This appears to be related to the fact that the mutant adhered to cecal mucus far better, but penetrated mucus less well than the wild-type, or even nonflagellated or nonchemotactic transductants (137). This also supports the idea that flagellar motility is not important in mobility within mucus.

The other major mechanism bacteria use to remain in the intestinal tract is adhesion. Piliated *Escherichia coli* are able to bind to sugar residues in the glycoproteins and glycolipids of both epithelial cells and mucus. In enterotoxigenic *E. coli* strains, K99 fimbriae bind to galactose (155) and sialic acid residues (119), K88ab fimbriae bind D-galactosamine residues (143), and the terminal subunit of F17 fimbriae binds to undetermined carbohydrate sidechains which are also present in cow plasma glycoproteins and hen egg white (195). The F-18 colicin which is found in nonpathogenic *E. coli* binds to mannose (243). Although *E. coli* lacking F-18 are able to colonize streptomycin-treated mouse large intestine when given alone, they cannot compete with the fimbriated bacteria in establishing infection when given concurrently (244).

Both piliated and non-piliated strains of enteropathogenic *E. coli* adhere to mucus (259). Denaturation, trypsinization, or removal of carbohydrate from the mucin all decrease binding (248). More mucin bound to both enteropathogenic and enterotoxigenic *E. coli* strains at pH 5.7

than at pH 7.4 (248). *In vitro*, the presence of mucus competitively inhibits binding of *E. coli* to colonic epithelium (125), suggesting a protective effect. On the other hand, adherence to mucus may allow for the initial colonization by bacteria. Hydrophobicity of the mannose-resistant AF/R1 pilus of the enteropathogenic *E. coli* strain RDEC plays a major role in bacterial binding to mucus and membranes (37), and RDEC preferentially colonizes colons of weanling rabbits, where mucus is more hydrophobic than in sucklings or in the ileum (124). Additionally, more mucus binding is seen in weanlings than in adolescents (248), which may reflect the increased susceptibility of weanlings to clinical disease.

Virulent strains of *Yersinia enterocolitica* are able to colonize the intestine because the *yadA* gene on the virulence plasmid codes for a high molecular weight outer membrane protein which allows binding to mucin (170). However, preincubation with mucus decreases the adherence of the organism to brush border membranes (130). It appears that coating with mucus changes the bacterial surface from hydrophobic to hydrophilic, decreasing the interaction of the organism with the epithelial surface (170). However, strains containing the virulence plasmid are able to degrade mucin to a greater extent than non-virulent strains (132), and thus overcome the protection afforded by mucus.

Helicobacter pylori has also been shown to bind to mucin, and the binding is decreased after the removal of sialic acid (237). Electron microscopy shows that most strains of *Vibrio cholerae* bind preferentially to mucus, rather than to the epithelial surface (260, 261). Guinea pig mucus inhibits invasion of epithelial cells by *Shigella flexneri*, but monkey mucus does not (245).

Intestinal mucins inhibit replication of rotavirus *in vitro* (24, 262). The rotavirus vp4 protein, which also mediates binding to cells, is involved in the binding (24); however it is not clear if sialic acid residues or short oligosaccharide chains act as the mucin receptor (262).

Mucus also plays a significant role in parasitic infections. Frick and Ackert (54) found that duodenal mucus of adult chickens inhibits the growth of *Ascaridia galli* more than that of young birds, and that this may play a role in age resistance. A nematode which has been studied more extensively is *Nippostrongylus brasiliensis* in rats. Following a transient decrease, an increase in the number of goblet cells is associated with an immune-mediated expulsion of this worm. If the number of goblet cells is decreased secondary to a protein deficient diet, the efficiency of expulsion is decreased (249). Secretion of mucus is necessary for the expulsion event: if mustard oil or a combination of cysteine and papain is administered to immune rats to cause secretion of stored mucin 1.5 hours prior to challenge, then

worm expulsion is inhibited (146). The role of prostaglandins in this process is unclear. While some investigators have found that administration of prostaglandins enhances expulsion, others have been unable to confirm this (28). The vehicles used (chloroform or alcohol), or the timing of administration may be responsible for the discrepancy.

Some worms are entrapped by mucus prior to expulsion (145). In addition, in a phenomenon called "immune exclusion", worms are unable to penetrate the mucus of immunized animals, while in naive animals, worms are able to burrow through the mucus to the intervillous space (145). Similarly, it has been shown that mucus from infected sheep inhibits the motility of *Trichostrongylus colubriformis* in agar gel (103). *Nippostrongylus* is able to move through viscous gels by forming a tight corkscrew (111); the presence of coating antibodies may prevent this motion, resulting in immune exclusion. It has also been shown that the worms ingest mucin, and morphologic damage of the adult worm gut is associated with the development of immunity (145).

Trichinella spiralis is expelled from immunized rats in a very similar manner, although *Trichinellae* enter the epithelium, while *Nippostrongylus* remain between villi (145). It has been shown that antibody or complement coating of *Trichinella* larvae allows entrapment in mucus (18, 145). This may be important in the expulsion of worms from the organisms, although the injection of antibody allows for the expulsion of

worms from the infected epithelium, prior to association with mucus.

The expulsion of both *Trichinella* and *Nippostrongylus* is an immune-mediated event. Transfer of thoracic duct lymphocytes from immunized rats results in accelerated expulsion of *Nippostrongylus* associated with early goblet cell hyperplasia (147), and this was later shown to be T cell-dependent (145). However, transfer of hyperimmune serum will also result in rapid expulsion (145). Furthermore, at least two steps are involved in worm expulsion (88). The first involves T-cell dependent "damage" to the worms. These damaged worms can then stimulate alterations in the terminal sugar residues of mucus resulting in the selective expulsion of damaged or healthy worms, even from athymic rats (88). There is also synergistic interaction between immune serum and thoracic duct lymphocytes in the rapid expulsion of *Trichinella* (1). In both worms there are increased concentrations of leukotrienes and rat mast cell protease II (145, 150). Corticosteroids, which inhibit the immune response in many ways and also decrease mucus secretion (141), delay expulsion of *Nippostrongylus* (145) as well as the rat tapeworm *Hymenolepis diminuta* (139). Reserpine also alters mucus secretion (169) and delays expulsion of *Nippostrongylus* (145).

On the other hand, mucus can apparently increase pathogenicity of other parasites. In particular, it promotes

survival of *Giardia lamblia* in many ways. For example, *Giardia* can be killed by the lipolytic products of milk, but this killing is inhibited by the presence of intestinal mucus (265). Mucus provides a nutrient source for the parasite (57), and allows for its enhanced adherence to the intestinal epithelium (145). This lectin-like adhesion to simple sugar residues within mucus, especially sialic acid, is a characteristic of several protozoa, including *Entamoeba histolytica* (145) and *Tritrichomonas mobilensis* (33). The yeast *Candida albicans* has also been shown to associate with mucus, but the ability to colonize the intestinal mucosa is dependent on the absence of normal flora (102).

Nutrient for Flora

Evidence that mucus may aid the growth of bacteria was demonstrated by Smith *et al.* (213, 214), who found increased virulence of intraperitoneally injected bacteria (*Staphylococcus aureus* and *Streptococcus* species) when mucus was administered with the organism. This effect was partially due to sequestration of the bacteria from the immune system. However, the chemical components of the mucus itself increased the growth of the organisms, presumably by serving as a nutrient source. It has been shown *in vitro* that jejunal mucus stimulates the growth of *Giardia lamblia* (57). Mucin can also enhance growth of both virulent and avirulent *Yersinia enterocolitica*, as well as serve as a nutrient source

for *E. coli, Salmonella typhimurium, Clostridium perfringens, Bacteroides* sp, *Shigella flexneri, Rumenococcus,* and *Bifidobacterium* (8, 132).

By comparing the cecal mucus of conventional and germfree rats, Lindstedt *et al.* (120) were able to show that normal flora degrade a significant amount of mucus. There was less mucus in the cecum of conventional animals, and a higher proportion of what was there was of lower molecular weight, indicating partial degradation. Other investigators have confirmed these findings (52). However in the colon, Szentkuti *et al.* (225) found a thinner mucus layer in germfree rats, associated with decreased mucosal thickness, and decreased numbers of goblet cells.

As discussed previously, *Rumenococcus* and *Bifidobacterium* species possess glycosidases that are responsible for the degradation of mucin in humans. The monosaccharides released from mucin by these glycosidases support growth of *Bacteroides* and other fecal bacteria that lack such glycosidases (82). However, a strain of *Bacteroides vulgatus* isolated from patients with Crohn's disease is capable of degrading mucus glycoproteins (192). The enzymes produced by normal flora may also directly attack pathogenic bacteria (160); normal flora certainly inhibit colonization by pathogens, since antibiotics increase infection with pathogenic organisms (245).

Diffusion Barrier

Much of the cytoprotective action of the mucus layer has been attributed to the barrier properties of mucus (79). However, when this property was experimentally examined, Heatley illustrated that mucus is not a barrier to the passage of solutes, such as H^+ ions and pepsin. Furthermore, when studies were performed to determine the thickness of the "unstirred water layer" in the intestine, the calculated value for several solutes was similar, so it was proposed that mucus merely acts as a support for the unstirred water layer (215, 250). However, more recent studies show that mucus does retard diffusion of many molecules. For instance, it slows the passage of H^+ ions relative to their rate of diffusion in water (172, 253), and Lucas (122) demonstrated that this retardation is dependent on the concentration of mucus. The mobilities of sodium, potassium, and chloride are also greatly reduced in mucus (68).

Mucus forms a polyanionic gel, and thus acts as an ion exchange resin (48). In general, chloride is excluded, whereas there is a high affinity for calcium and potassium (67, 199). However, although the transport of chloride (and other ions) is retarded, it is not prevented (68). Sequestration of potassium by mucus and cell surface glycoproteins may be important in recycling of potassium by the Na^+/K^+-ATPase. Calcium may be important in the

condensation of mucus into compact granules prior to exocytosis (242).

Mucus retards diffusion of molecules as well as ions. Smith (212) found a diffusion coefficient for butyrate in mucus to be 50-60% of that in water. Desai *et al.* (34) determined the diffusion coefficients for a wide variety of molecules in mucus and concluded that no consistent effect of molecular weight was evident with regard to barrier properties for the weight range tested (34-660 Da). Using cultured goblet cells, it has been shown that the overlying mucus layer is a significant barrier to the passive absorption of the lipophilic and uncharged drug testosterone (97). Mucus has been shown to trap iron, but it is not clear if this aids (181), or prevents excess, (252) absorption. This trapping may make iron more available to bacteria (51).

Since mucus is a polyelectrolyte gel, it behaves as a Donnan system (67). Consequently, the hydration of mucus is dependent on the ionic concentrations in the bathing solution. As ionic strength is decreased, hydration is increased, and noncovalent interactions between mucin subunits are fewer, decreasing viscosity. Conversely, at high ionic strength, anionic charges within the gel will be shielded, and hydration and volume decreased. It has been postulated that lack of functional chloride channels resulting in decreased chloride movement in cystic fibrosis could alter ionic composition resulting in abnormal mucus secretions in intestinal,

pancreatic, and respiratory tissues (138, 242). Mucus from cystic fibrosis patients is hyperpermeable to small ions and water, associated with an increased calcium content (58). Furthermore, glucose absorption was enhanced in afflicted patients, coinciding with a decreased thickness of the calculated unstirred water layer (53).

Mucus may even have a "waterproofing" function (159). If the external mucus layer is removed from an eel, the weight of the animal will increase when the eel is placed in distilled water. This property has not been examined in the intestine, and would be difficult to address in light of the complex absorptive and secretory processes involved in digestion and absorption. However, it is interesting that Westergaard and Dietschy (250) saw swelling of intestinal villi inversely correlated the thickness of the unstirred water layer.

Creation of Microenvironment

A microenvironment occurs when the concentration of solutes next to a membrane is different from that of the bulk phase on either side of the membrane, as has been shown to occur in mucus (67). In the gastrointestinal tract, the presence of a microenvironment is important in two basic areas. In the stomach, it is necessary to prevent direct contact between the highly acidic bulk contents and the mucosal surface. When Heatley (75) found that mucus did not act as a barrier for hydrogen ions, he proposed a pH gradient

within the mucus layer. In his dynamic model, mucus and bicarbonate are secreted at the mucosal surface, and then migrate outward. As the mucus progresses into the lumen, it becomes more hydrated and accumulates more hydrogen ions. The viscosity thus decreases, until the outermost layer is shed into the lumen. Therefore, some of the hydrogen ions will be brought back into the lumen with the dissolving mucus, while others will be neutralized by the bicarbonate within.

This "mucus-bicarbonate barrier" model has stood the test of time very well (5, 31, 148, 205). With the development of microelectrode techniques, pH gradients have been demonstrated in rat (190), rabbit (254), and human (182) gastric mucus *in vivo*. The pH gradient can be decreased with the addition of compounds that dissolve mucus, such as N-acetylcysteine (190).

Mucus is responsible for the creation, as well as the support, of a microenvironment in the intestine. Hogben *et al.* (78) proposed that an acidic microenvironment could influence the absorption of drugs from the intestinal tract. Acidic drugs are absorbed more rapidly than would be expected at neutral pH; however at acidic pH these acids would be undissociated, and would readily cross the epithelial membrane by nonionic diffusion. He calculated that the pH at the rat jejunal surface must be 5.3 to account for measured drug absorption rates. Surface pH measurements with microelectrodes have revealed an acidic microenvironment, but with pH 6-7 (123, 185, 203).

Similarly, a slightly acidic microenvironment is present in the colonic unstirred layer (185). This would allow for the passive absorption of butyrate and other short chain fatty acids in the undissociated form (7). Although there is evidence for a bicarbonate gradient-dependent, carrier-mediated anion exchange process for butyrate in the colon, this cannot account for the rate of absorption measured experimentally, nor could passive diffusion of the dissociated ionized form of butyrate (136). In the rodent cecum, the microenvironment is more basic than the luminal contents, and the pH may have an effect on the virulence of *Entamoeba histolytica* (112). This organism is subject to killing by ammonia, and species with a higher cecal mucosal pH, such as the rat, are less susceptible to infection than those with a lower pH, such as the gerbil.

Techniques Used to Study Mucus

Model Systems

Intestinal loops. For *in vivo* experimentation of the gastrointestinal tract, it is common to isolate the part of the tract under study to minimize effects of the rest of the system, and to allow for easy access. For the study of gastric secretion, the first widely used technique was formation of the Heidenhain pouch (76). Florey (44, 45, 46, 47) used a variety of intestinal pouches, blind loops, and transplanted segments to examine movement of mucus within the

intestine, and the effects of feeding, neural stimulation, and various chemicals on mucus secretion.

Explants. Mucosal samples (161) and full thickness intestinal explants (50) have been used to measure secretagogue activity *in vitro*. These tissue sections can be maintained in culture medium for several hours, and mucus production and secretion can be measured by several methods. This technique allows for concurrent testing of several different chemicals on tissues from a single animal. It has even been used to demonstrate goblet cell secretion following electrical field stimulation, due to intrinsic nerve stimulation (175).

HT-29 cloned goblet cells. Until recently, it was impossible to work with isolated goblet cells, because these cells lose their polarity and ability to secrete when separated from the mucosal epithelium (160). However, cell culture systems are now available. The HT29 colon adenocarcinoma cell line is undifferentiated under standard culture conditions. However, substitution of glucose with galactose (83), or long term treatment with butyrate (108) results in differentiation of cells. Individual cells can be selected for cloning. Several sublines, including HT29-18N2 and Cl.16E, show characteristics of goblet cells, including baseline mucus secretion, and the ability to respond to cholinergic stimulation (108, 173).

Quantitation of Mucus

Biochemical techniques. Biochemical measurement of total protein-bound hexose (258), acid-precipitable protein (50), or glycoprotein (127) has been used to estimate mucus concentration; however mucins are not the only glycoproteins present in the intestine. Mucin can be separated from many other glycoproteins by gel filtration on a Sepharose-4B column, but many proteins remain associated with the sticky mucin (160).

Radioactivity incorporation. Glycoprotein synthesis has been measured by determining the rate of ^{14}C- or ^{3}H-glucosamine incorporation into intestinal slices (50), and secretion is measured by counting precipitable radioactivity released into the media (49). By fractionating the cell sap on a Sepharose-4B column, and collecting the void volume, it is possible to estimate the amount of mucin synthesized. It has also been shown that labelled butyrate and acetate are incorporated into mucus glycoproteins (29). However, absorptive cells synthesize and release glycoproteins at a faster rate than goblet cells (160).

Morphometry. Goblet cell hyperplasia, which is associated with increased mucus secretion, can be quantitated morphometrically (147). Compound exocytosis can be visualized, and the percentage of cavitated goblet cells is reflective of the amount of mucus secreted (217).

Immunologic techniques. Standard radioimmunoassay (131) and enzyme-linked immunosorbant assay (ELISA) techniques (188) have been developed, using antibodies to mucin. Polyclonal antibodies react predominantly to the "naked" regions of mucin, and therefore do not measure degraded mucin (131). Monoclonal antibodies have been made specific for both the naked peptide backbone, and for intact mucin (210). Antibodies have also been made which are specific for the fragment known as the "link" glycopeptide (188).

Lectin binding techniques. An enzyme-linked lectin assay (ELLA), very similar to a standard ELISA, has been developed (27, 69). The lectin soybean agglutinin preferentially binds to N-acetylgalactosamine residues, which are abundant in mucin but less common in other glycoproteins.

Secretion of Mucus

Light microscopy. A monoclonal antibody has been developed which specifically labels goblet cells of the human colon, appendix, and small intestine (240). This may be useful in the diagnosis of disease states, in which the quantity or quality of mucus is altered. Histologic examination of the thickness of the mucus coating can be used to evaluate changes in secretion (225).

Morphometry has also been used to quantitate secretion by comparing the amount of stained mucin in goblet cells before and after addition of a secretagogue (100). Autoradiography

has been used to measure the rate of secretion (73). Cavitation of goblet cells indicates that compound exocytosis has taken place, and has been used to measure secretagogue activity (176, 177, 217). Exocytosis has been directly observed by video-enhanced light microscopy (230).

Electron microscopy. The steps of the secretory process can best be followed by electron microscopy (217). Since conventional fixation techniques can fragment the limiting membranes, cryofixation may allow for better evaluation of the mechanism of secretion (84). The thickness and composition of the mucus layer can also be examined by this technique (165).

Composition of Mucus

Biochemistry. Standard biochemical techniques have been used to determine the amino acid and sugar composition of mucus (160). Glycosyl-transferase activities can be measured by conventional biochemical assays (12, 226), providing clues to changes in carbohydrate composition. Methods for measuring the adhesiveness, plasticity, viscoelasticity, and spinnability of mucus microsamples are also now available (263).

Histochemistry. Differential staining techniques can be used to distinguish mucin characteristics. When sections are stained with combined alcian blue and periodic acid Schiff reagent, acidic mucins will appear blue, and neutral mucins will appear red (225). High iron diamine will stain sulfated

sialomucins (266). Positive staining with mild periodic acid Schiff (mPAS) indicates deficiency of O-acetylation of sialic acid, as is seen in ulcerative colitis (94). Mucins of different chemical composition can be distinguished by differential lectin binding. Therefore, lectin histochemistry can be used to identify abnormally glycosylated mucins in goblet cells, which may be associated with disease (89, 241), developmental changes (227), or diet (152). Finally, specific antibodies can be used to distinguish small intestinal from large intestinal mucins by indirect immunoperoxidase staining (43).

Diffusion Through Mucus

Mucus can be immobilized on a filter, or between two filters, and the rate of diffusion through this compared to the rate of diffusion through the filter alone (75). The mathematical modeling of diffusion through mucus in this type of apparatus has been described (172). Diffusion through mucus overlying HT29 monolayers can also be measured (97).

Microbial Virulence

In vivo colonization assays, where the number of colony-forming units of bacteria recovered in the feces after experimental infection is measured over time, reflect a combination of virulence factors (162). Microbial adherence can be evaluated by coating plates with mucus or brush border

membranes, and measuring the percentage of radiolabelled bacteria which will then adhere (130). Competition for binding can also be measured with this assay, by adding the competitor along with the organism. Adhesion of organisms to cells in culture can also be evaluated microscopically (33). Penetration of organisms through mucus can be evaluated by the passage of radiolabelled bacteria through a mucus gel (170), or by direct observation of the organism (42, 111).

Theoretical Considerations

Lubrication

The idea that mucus is necessary for lubrication to aid in the passage of feces has been mostly intuitive, with little direct experimental support. However, there is one disease state which illustrates the need for such lubrication quite clearly. In cystic fibrosis, where mucus is abnormally viscous and lacks elasticity, it is common for infants to develop meconium ileus, and for adults to develop meconium ileus equivalent (160). As indirect evidence, the most compact, continuous mucus layer is in the distal colon, where there is greatest need for lubrication for the passage of formed feces (194). In addition, the fact that nondigestible solids are coated in mucus when they are expelled from the stomach suggests that mucus aids in lubrication to facilitate their passage through the pyloric sphincter and expel them from the body (66).

Cytoprotection

Mucin itself has no direct buffering or antienzymatic activities. However, its presence is necessary to protect underlying cells. Its removal may result in increased gastric or duodenal ulceration (104). Chronic inflammatory bowel disease is associated with decreased mucus production, which may help to perpetuate the disease (160). This protection is partly due to the role of mucus as a diffusion barrier, and the creation of a microenvironment, which will be discussed below. In addition, it appears that mucus is able to detoxify reactive oxygen intermediates (34, 62). Irritants of many types stimulate the accelerated secretion of mucus; this may be a protective mechanism to keep such irritants away from the intestinal mucosa (21, 47).

Protection from Infection

The interactions of mucus and pathogenic organisms are complex. It is perhaps easier to determine the protective effects of mucus by looking at mechanisms that pathogens have developed to overcome these effects. Mucus is a slippery substance, and when the motility of the intestine is taken into account, it is clear that attachment to mucus is necessary if organisms are to avoid being flushed out. The viscosity of mucus is also a barrier, and motility based on internal structures rather than flagellae helps microorganisms

negotiate this barrier (42). That organisms have developed mucinase enzymes indicates that mucus is a barrier that must be negotiated (168).

Mucus also contains antibacterial substances, in particular lysozyme (61) and secretory IgA (140). Mucin bound to IgA allows for entrapment of parasites such as *Nippostrongylus*, so that the parasite is immobilized and swept out of the body (145). The goblet cell hyperplasia seen with many parasitic infections emphasizes the importance of mucus in their expulsion. Mucus coating can also prevent attachment of pathogens to the intestinal epithelium by competition (125) or by changing bacterial surface properties (170).

Pathogenic organisms have also been able to use mucus to increase virulence. Within mucus, bacteria are "hidden" from some of the defense mechanisms of the host (265). Also, mucus can provide nutrients to these organisms, as discussed below.

Nutrient for Flora

Although the experimental evidence is incomplete, it seems reasonable that degraded mucus provides nutrients for bacterial flora, especially in times of fasting. The trapping of iron by mucus (181, 252) allows the flora increased access to this mineral which is so tightly controlled in the rest of the body. It is important to remember that the normal flora also provide a major defense against pathogenic bacteria (160,

245), so that in providing nutrients for flora, mucus is actually protecting the host from infection.

Diffusion Barrier

The gastrointestinal mucus layer has been shown to delay diffusion of a wide variety of molecules. In the intestine, this layer is between 50 and 500 μm thick, depending on species and site evaluated (34, 67). In general, the measured unstirred water layer is slightly thicker. This may be due to an unstirred layer overlying the mucus, but is more likely due to the decreased diffusion of solutes in mucus leading to erroneously large values for the unstirred water layer (215). Consequently the barrier to nutrient absorption is formidable, although the calculated maximum allowable water barrier which would allow physiological absorption of glucose and lipid is 40 μm (223). Furthermore, the mucus layer is not static; there is constant renewal at the mucosal surface and degradation at the luminal surface, resulting in a net flow of mucus away from the mucosa.

It would seem that this unstirred mucus layer would make absorption of nutrients impossible. But on further examination, the term "unstirred" is also inappropriate. The mixing caused by segmental and peristaltic contractions of the intestine, with three dimensional shear forces, as well as expansion and contraction of the mucus layer itself, is not comparable to mixing by a stir bar in a beaker. Furthermore,

the intestinal villi move in and out of the mucus layer, cleansing themselves of particulate matter, and moving the entire mucus layer aborad (46). Villi bare of mucin are present in the small intestine, particularly the ileum, *in vivo* (165). The villi may also cause mixing within the mucus layer, as speculated by Strocchi and Levitt (223).

The fluxes of water in and out of the intestine must also be considered. Large volumes (approximately 1 liter/20cm/hr in a human, 53) are secreted from the crypts and absorbed from the villi. This could aid in absorption by solvent drag effects, as well as contribute to mixing within the mucus layer. And finally, the mucus may actually "trap" certain molecules, such that the concentration within the mucus can exceed the luminal concentration, leading to increased absorption.

Creation of Microenvironment

The pH difference between the lumen and the mucosal surface is most important in the stomach, where the presence of a "mucus-bicarbonate" barrier is generally accepted. By delaying the diffusion of H^+ toward the epithelial cells, and the diffusion of bicarbonate away from them, mucus plays a vital role. The microenvironment may also play a role in the selective absorption of drugs and nutrients from the intestinal tract. In particular, a slightly acidic

microenvironment would aid in colonic absorption of short chain fatty acids (7).

Conclusions

The differences in the mucus layer throughout the gastrointestinal tract reflect the differing functions required. In the stomach, the major function is cytoprotection, by creation of a diffusion barrier and a less acidic microenvironment. For this, the mucus is neutral rather than acidic, and is more viscous. In the small intestine, mucus must act as a diffusion barrier to destructive enzymes and pathogenic organisms, yet provide a microenvironment to allow for absorption of nutrients. The fact that absorption occurs at the tips of the villi, which stick up through the mucus layer, may be a physiologic necessity, rather than a random fact for students to memorize.

In the proximal colon, the loose, discontinuous mucous layer provides for the nutrition of friendly flora, and creates a microenvironment which allows for absorption of the short chain fatty acids produced by these bacteria. Finally, in the distal colon, a thin, compact mucus layer provides lubrication for the passage of feces. All in all, mucus aids the host in a number of ways, and its importance in the maintenance of good health is often overlooked.

CHAPTER 3
PILOT STUDIES

Introduction

The overall purpose of this project was to determine if there is a mucus secretagogue in the cecal contents of rabbits with mucoid enteropathy, as proposed by Toofanian and Targowski (236). Therefore pilot studies were performed to: 1) explore the presence of such a secretagogue in the cecal contents of a rabbit with naturally-occurring mucoid enteropathy (ME); and, 2) confirm that the cecal ligation model developed by Toofanian and Targowski (229, 235) would reproducibly cause a mucoid enteropathy-like syndrome.

Cecal Filtrate Collection

Cecal contents were collected from a juvenile female New Zealand White (NZW) rabbit (*Oryctalagus cuniculus*) with naturally-occurring mucoid enteropathy, and also from two healthy adult NZW rabbits. The affected rabbit also had a severe intestinal *Eimeria* infestation. For each rabbit, the volume of the contents was estimated, and an equal volume of Hank's Buffered Salt Solution (HBSS) was added. The slurry was centrifuged at 3000 rpm (700 x g), 4°C for 15 minutes. The supernatant was collected, and recentrifuged at 9500 rpm

(10,000 x g), 4°C for 30 minutes. The resulting supernatant was then filtered through a Whatman #50 paper filter, followed by a 0.8 μm membrane filter, and finally a 0.22 μm sterilizing membrane filter. The sterile filtrate was stored at -70°C until use.

Intestinal Explants

The cecal filtrates that were collected were tested for mucus secretagogue activity in an *in vitro* intestinal explant system. Five healthy adult NZW rabbits were sedated with ketamine/xylazine, and killed with an overdose of barbiturate. Intestinal segments were collected, opened along the mesenteric border, and rinsed in HBSS, containing 100 μg/ml gentamicin, to remove ingesta. A 6 mm diameter Baker's biopsy punch (Baker Cummins Pharmaceuticals, Inc., Miami, FL) was used to take explants from the ileum and proximal colon (approximately 10 cm from the cecocolic junction, at the point where the number of longitudinal bands (teniae) decreases from 3 to 1, fig. 3.1). Longitudinally paired punches were taken for control and experimental samples, as the concentration of goblet cells varies along the length of the colon (236). Explants were incubated in 24 well polystyrene microtiter plates containing 0.5 ml of Trowell's T8 medium (GIBCO BRL, Gaithersburg, MD) with 100 μg/ml gentamicin, and 0.5 ml of the appropriate cecal filtrate in each well. The plates were incubated for 1 hour at 37°C in a 95%O_2/5%CO_2 humidified

environment. Following incubation, the culture medium was removed and frozen, while the explants were fixed in 10% neutral buffered formalin. Following routine processing and sectioning, the tissues were examined histologically. After 1, 2, and 3 hours of incubation, the tissues appeared viable in every respect.

Enzyme-Linked Lectin Assay

Quantitation of mucus release by the explants was accomplished using an enzyme-linked lectin assay (ELLA) as described by Cohan *et al*. (27). Ninety-six well polystyrene microtiter plates were coated overnight at 4°C with 100 μl of serial dilutions of porcine gastric mucin standard or medium/filtrate mixture following explant incubation in 0.5M sodium carbonate buffer, pH 9.6. The plates were washed with phosphate buffered saline containing 0.5% Tween-20 (PBS-T20), and blocked with 5% fetal calf serum in PBS-T20 for 1 hour at 37°C. The plates were again washed, and incubated for 1 hour at 37°C with 100 μl/well of a 10μg/ml solution of a soybean agglutinin (*Glycine max*)-horseradish peroxidase conjugate, which specifically binds N-acetylgalactosamine residues. Plates were then washed a third time, and the substrate o-phenyl diamine (OPD) was added. After 10 minutes, the reaction was stopped with 100 μl of 4N H_2SO_4, and the optical density at 492 nm was read. The standard curves for porcine

gastric mucin were consistent from plate to plate (figure 3.2).

Data Analysis

After the filtrates were incubated with paired explants, the mucus secreted into the culture medium was quantitated by the ELLA described above. The effect of sample dilution on the amount of mucus in the medium/filtrate measured by the ELLA is illustrated in figure 3.3 for a control rabbit, and in figure 3.4 for the rabbit with naturally-occurring mucoid enteropathy. At the lower dilutions (higher concentrations) there were too many proteins in each sample competing with the mucus for a limited number of binding sites on the plate, resulting in falsely low mucus readings. At the higher dilutions, there seemed to be some appropriate dilutional effect; however the signal-to-noise ratio is decreased, creating a larger variance. The 1:64 dilution was selected for studies requiring quantitation of mucus.

Using the porcine gastric mucin standard curve for the same plate, the optical density readings were converted to apparent μg mucus per ml of medium/filtrate (figure 3.5). The amount of mucus in the medium/filtrate incubated without an explant was subtracted from each value to give the amount of mucus secreted. Since each explant was incubated in 1 ml medium/filtrate, this number was equivalent to ng mucus secreted per explant. Each value was then divided by the

weight of the explant to obtain nanograms mucus secreted per milligram of tissue (figure 3.6).

The amount of mucus in the medium/filtrates initially and following explant incubation varied with the source of the cecal filtrate (figure 3.5; Appendix A). Secretion from both ileal and colonic explants was significantly increased compared to control ($P < 0.05$) when incubated with the cecal filtrate from the rabbit with mucoid enteropathy (figure 3.6).

Cecal Ligation

To better study mucoid enteropathy, it is necessary to have a reproducible experimental model of the disease. Cecal ligation has been reported to cause a mucoid enteropathy-like syndrome in rabbits (229, 235). Surgery was performed on one adult, female, conventionally housed NZW rabbit (*Oryctalagus cuniculus*). The procedure was approved by the University of Florida Institutional Animal Care and Use Committee. The rabbit was anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine intramuscularly. A sterile field was prepared, and the abdomen was opened. A window was made in the mesentery adjacent to the cecum. Two adjacent ligatures of 2-0 silk were placed around the cecum just distal to the sacculus rotundus, preventing the flow of ingesta into and out of the cecum, but not obstructing the flow from the ileum to the colon (figure 3.7). The linea alba was closed with 3-0

chromic gut, and the skin was closed with 3-0 silk. Recovery was uneventful.

The rabbit was observed twice daily. By the first postoperative day the rabbit was eating small amounts, and passing dry feces. However, appetite and fecal output decreased on the second day. On the third day the rabbit was anorectic, appeared painful, and had mucoid diarrhea. Euthanasia and necropsy were performed.

At necropsy, there was perineal staining with greenish, liquid fecal material. There were mild subcutaneous hemorrhages at the incision site, but the sutures were intact, and there was no gross inflammation. The cecal contents and the body of the cecum distal to the sutures appeared normal. The ampulla coli and proximal 2-3 cm of the colon were grossly dilated with gas and fluid, although there was no mechanical obstruction. The colon contained a small amount of greenish fluid, with mucus adhering to the mucosa. The contents of the ileum were similar. The jejunum contained yellow fluid, with pockets of accumulation of clear, gelatinous mucus. On histologic examination there appeared to be mild goblet cell hyperplasia in the ileum and colon. These results are consistent with those described by Toofanian (229, 235), and suggest that this method provides a workable model to study mucoid enteropathy.

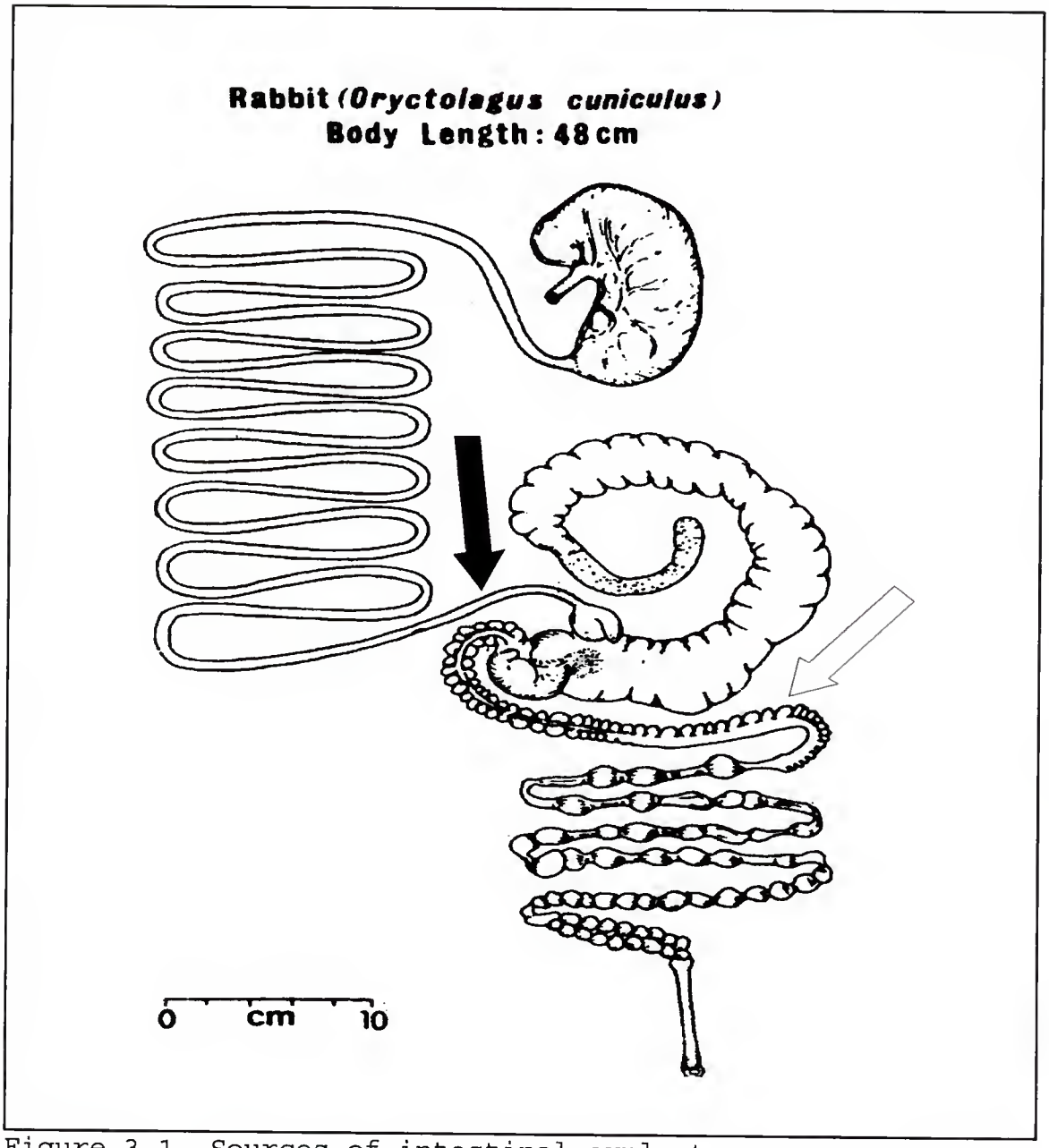

Figure 3.1. Sources of intestinal explants.

Explants were taken from the distal ileum (closed arrow) and the single-banded proximal colon (open arrow). Adapted from reference 6 (p. 270). Used by permission of the publisher, Cornell University Press.

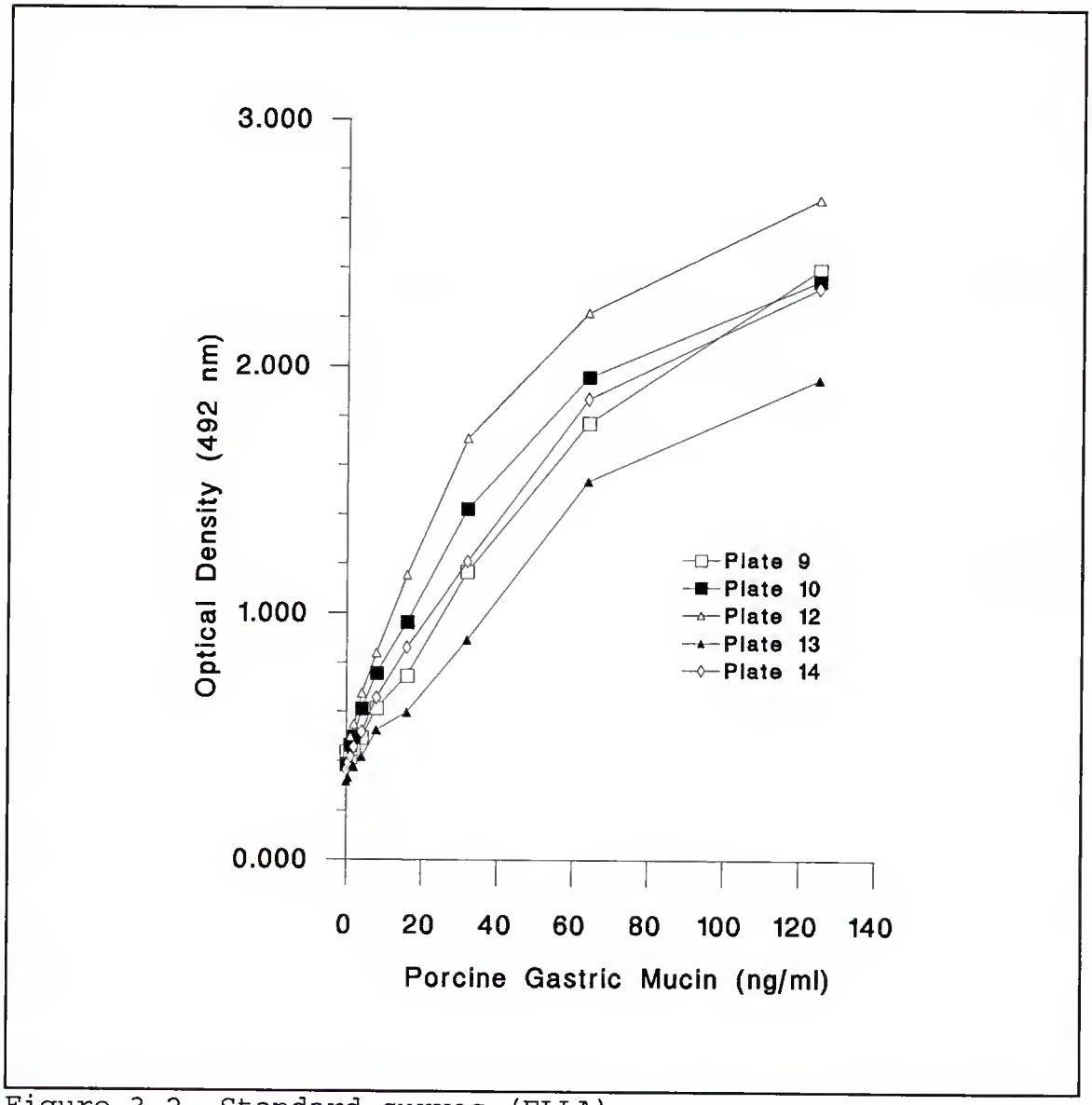

Figure 3.2. Standard curves (ELLA).

Serial dilutions of porcine gastric mucin analyzed by ELLA. The standard curves for the 5 plates used to collect data for this chapter are shown.

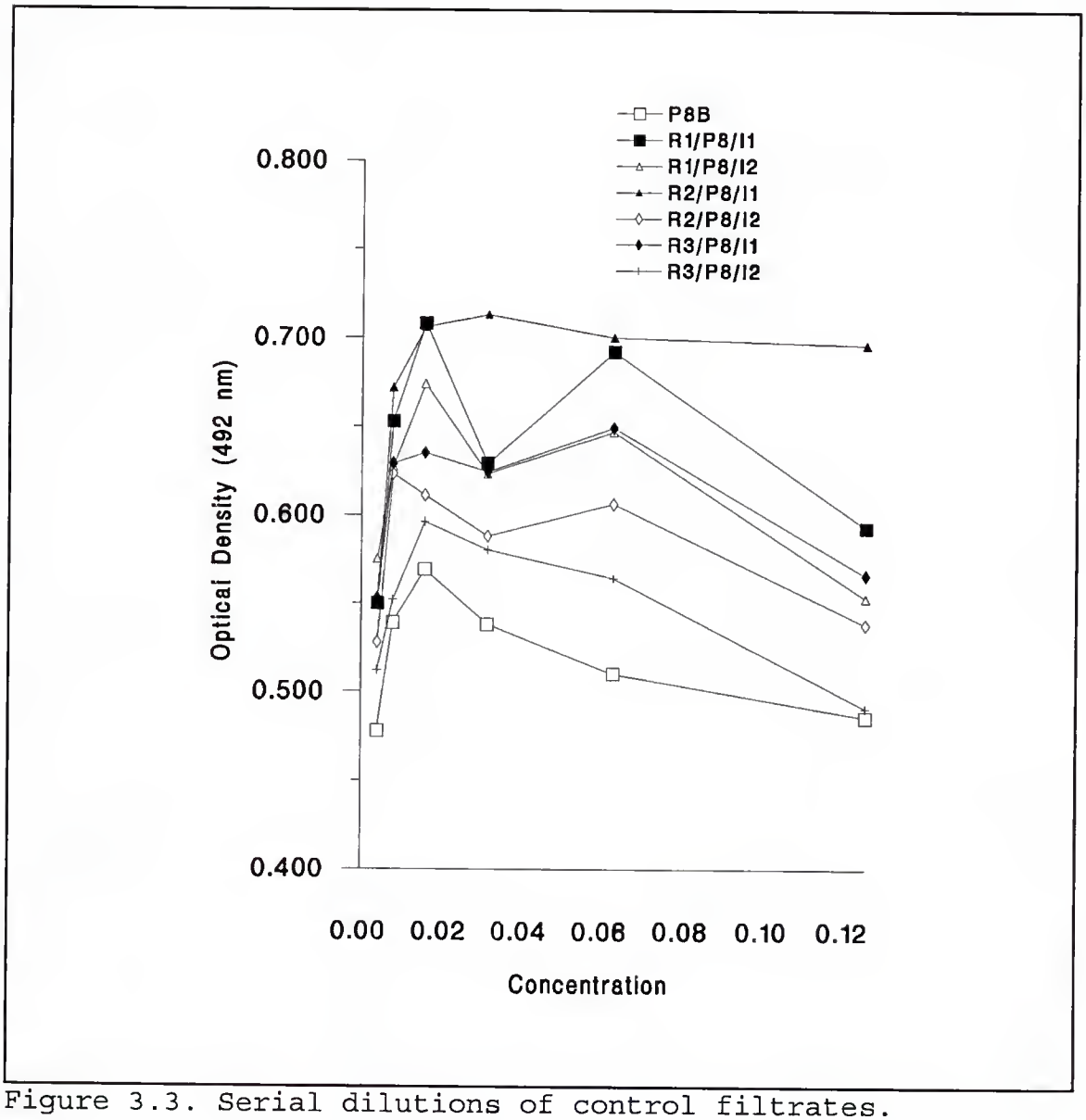

Figure 3.3. Serial dilutions of control filtrates.

Serial dilutions of medium/filtrate samples from a control rabbit (P8) incubated in the absence of an explant (P8B) or the presence of ileal explants from 3 rabbits (R1, R2, and R3), analyzed by ELLA.

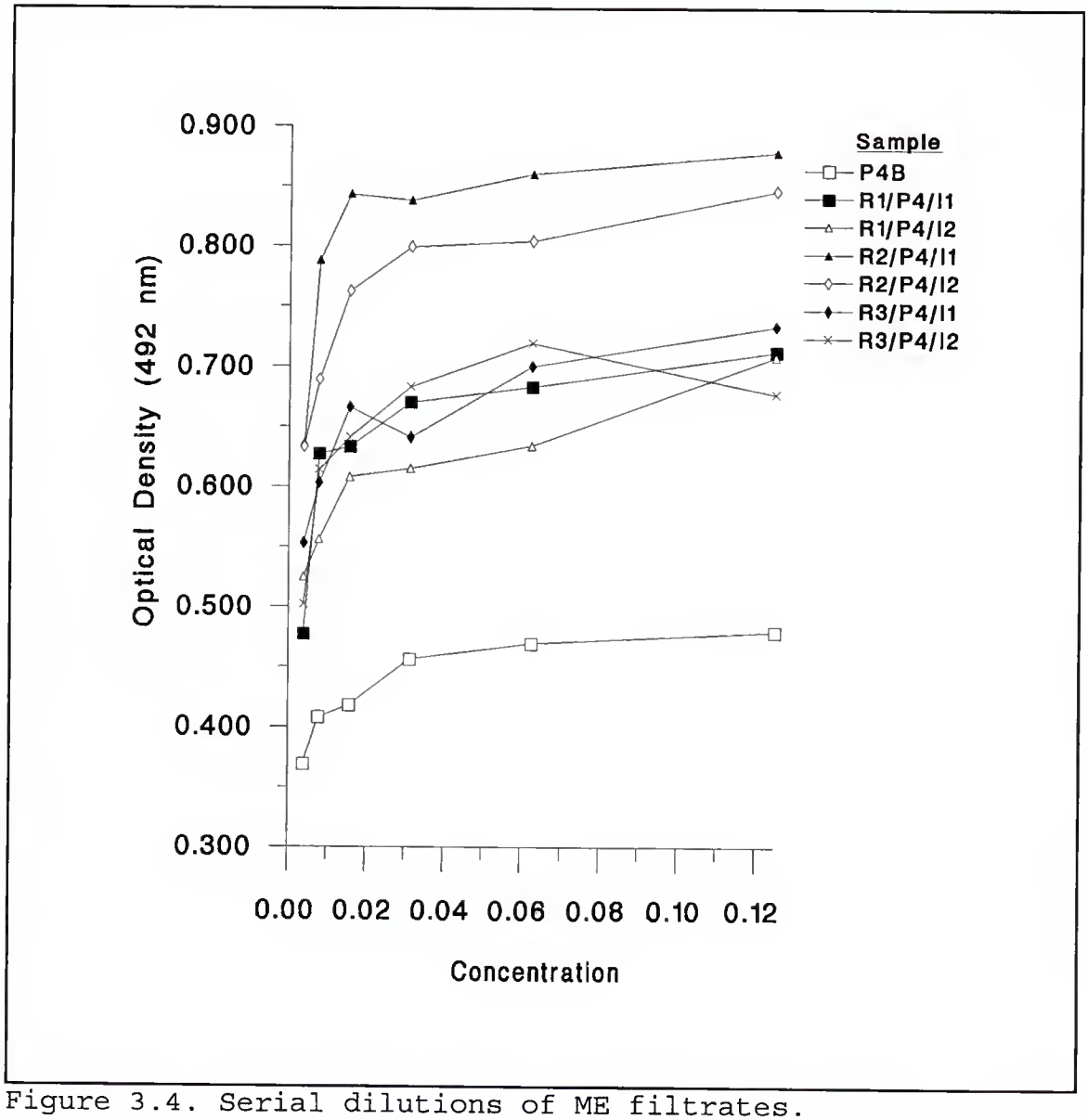

Figure 3.4. Serial dilutions of ME filtrates.

Serial dilutions of medium/filtrate samples from a rabbit with mucoid enteropathy (P4) incubated in the absence of an explant (P4B) or the presence of ileal explants from 3 rabbits (R1, R2, and R3), analyzed by ELLA.

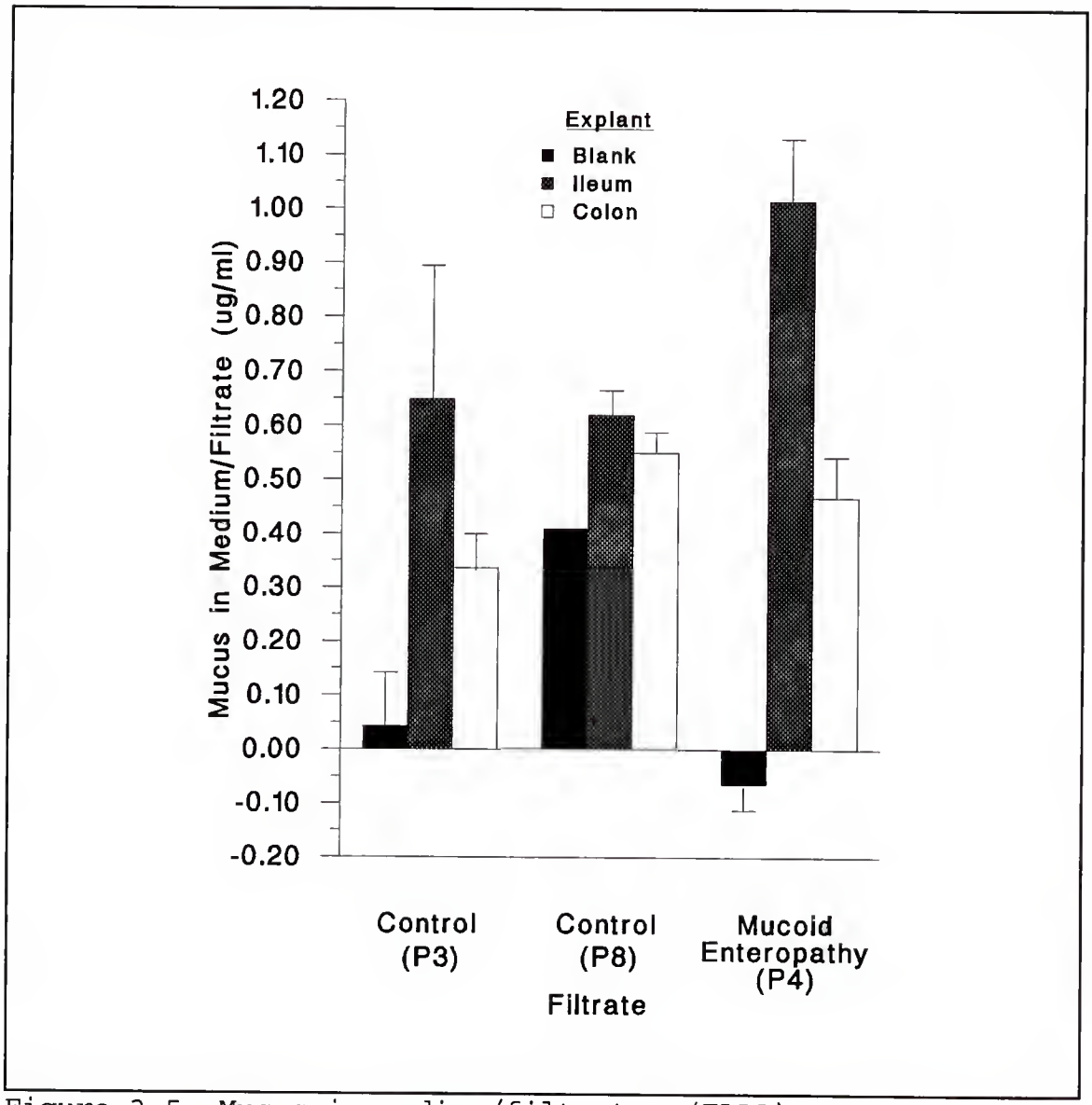

Figure 3.5. Mucus in medium/filtrates (ELLA).

Calculated mucus (porcine gastric mucin equivalents) in medium/filtrates, measured by ELLA. Bars represent mean + SEM. N=1 (P8 blank), 5 (P3, P4 blank), 6 (P8 ileum, colon), 7 (P3 ileum, colon), 10 (P4 colon), or 14 (P4 ileum).

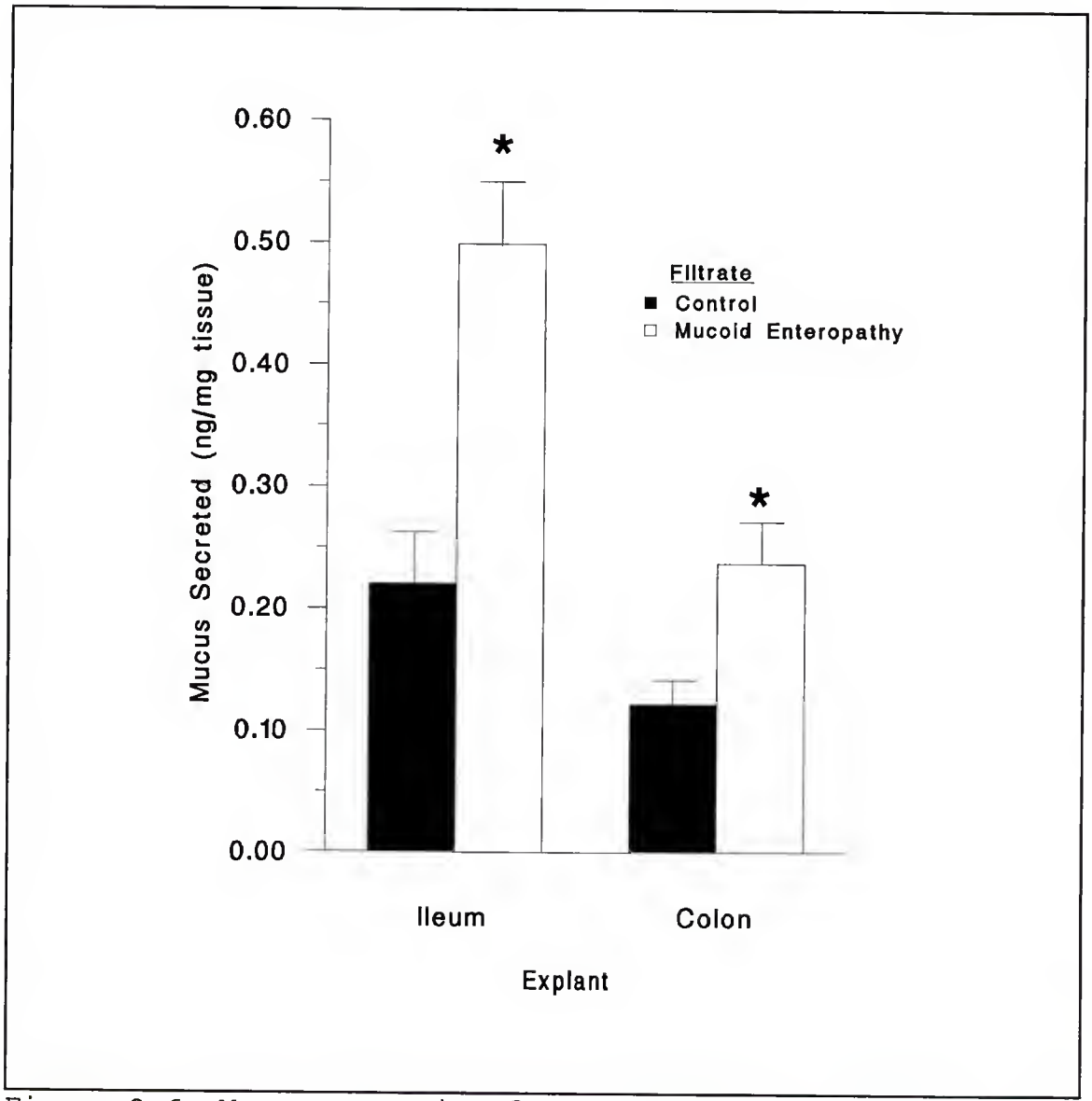

Figure 3.6. Mucus secretion from explants.

Mucus secretion was determined by subtracting the amount of mucus in the medium/filtrate incubated without explants (as measured by ELLA) from the amount incubated with explants. Values shown are relative to the wet weight of the explant. Bars represent mean + SEM. N = 8 (ME) or 11 (control). * = significantly different from control ($P < 0.05$).

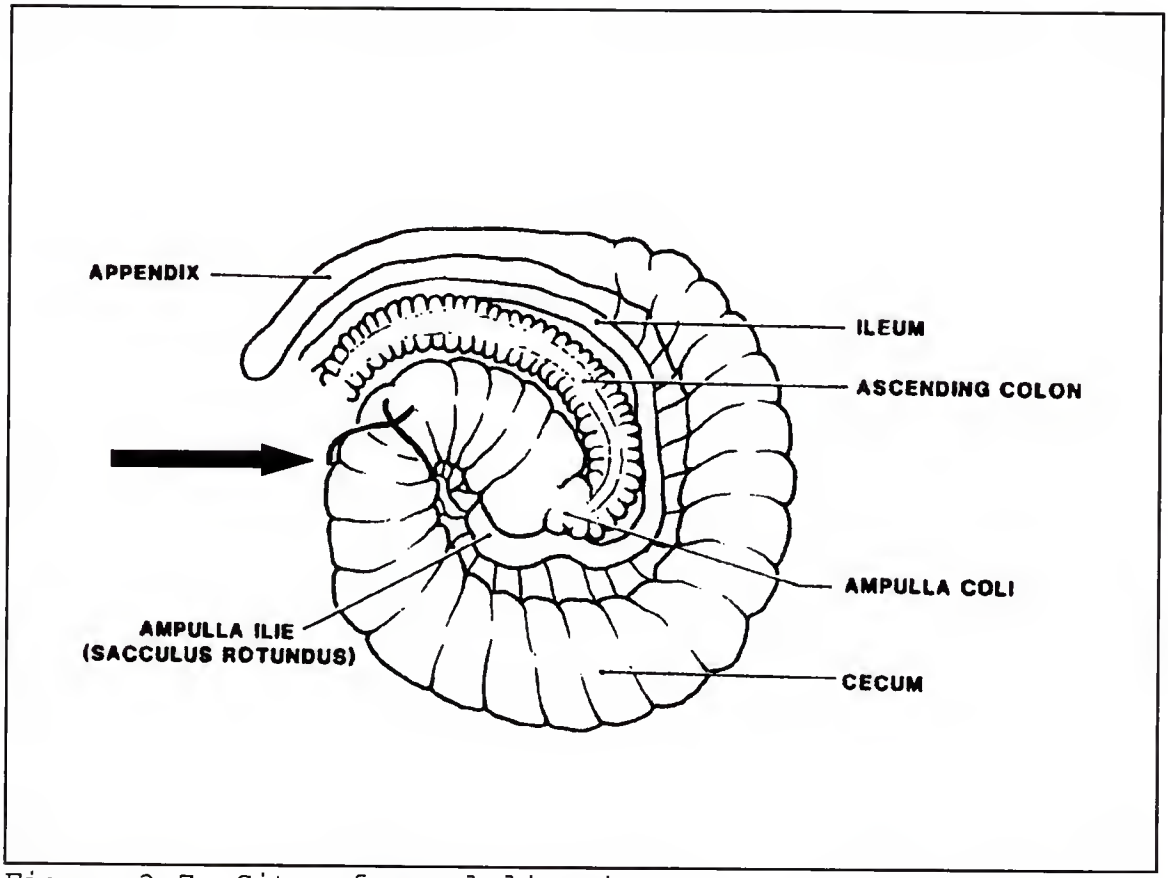

Figure 3.7. Site of cecal ligation.
The cecum was ligated distal to the sacculus rotundus (arrow), allowing the flow of ingesta from ileum to colon. Adapted from reference 235, with permission.

CHAPTER 4
REFINEMENT OF METHODS

Use of Soybean Agglutinin to Quantitate Mucus

Introduction

Soybean agglutinin, the lectin from *Glycine max*, specifically binds to N-acetylgalactosamine residues, which are a major constituent of the O-linked mucin glycoprotein, but are rare in N-linked glycoproteins. A direct enzyme-linked assay using this lectin for the measurement of mouse intestinal mucus has been described (27). Its suitability for the measurement of rabbit intestinal and colonic mucin was investigated using Western blot techniques.

Materials and Methods

Purification of rabbit colonic mucin. Colonic mucus from a rabbit with experimentally-induced mucoid enteropathy (rabbit H5, see chapter 5) was isolated by the method of Mantle and Allen (128) with minor modifications. Mucus gel was removed from the colonic mucosa with forceps, and was homogenized by hand in a Kontes tissue grinder with an equal volume of 5 mM EDTA and 1 mM PMSF. The suspension was centrifuged at 30,000 x g for 30 minutes. 8.1 g of cesium

chloride (CsCl) was added to 13 ml of the supernatant for a final concentration of 0.6 g/ml. This solution was centrifuged for 24 hours at 1.5 x 10^5 x g at 4°C in a Beckman L7 Ultracentrifuge with a 70.1Ti rotor. Eight 1.6 ml fractions were collected, and 100 μl of each was evaluated for carbohydrate content. The four densest fractions were pooled, and the volume was brought up to 13.5 ml with 0.6 g/ml CsCl, and the centrifugation was repeated, this time for 48 hours. Following carbohydrate quantitation, fractions 2 to 5 (from heaviest to lightest) were pooled, then concentrated and desalted using a Centricon®-100 concentrator (Amicon, Inc, Beverly, MA). This solution was applied to a 2.6 x 14 cm Sepharose 4B column. The fractions containing the void volume were pooled, dialyzed against distilled water, and frozen at -70°C. Porcine gastric mucin (Sigma Chemical Co., St. Louis, MO), was used as a mucin standard for comparative purposes.

Periodic acid-Schiff assay for carbohydrate quantitation. The method used was that of Mantle and Allen (127). Periodic acid solution was prepared by dissolving 25 mg of periodic acid in 7% acetic acid (3.5 ml glacial acetic acid in 50 ml distilled water. Each sample was brought up to a volume of 2.0 ml. 0.2 ml periodic acid solution was added to each sample and incubated for 2 hours at 37°C. Then 0.2 ml Modified Schiff reagent (Fisher Chemical, Pittsburgh, PA) was added, and samples were incubated 30 minutes at room temperature. The optical density at 555 nm was read.

Protein concentration. A kit (Sigma Chemical Co., St. Louis, MO) employing Peterson's modification of the micro Lowry method was used to measure total protein concentration. The kit was used according to the manufacturer's instructions.

Direct enzyme-linked lectin assay (ELLA). Porcine gastric mucin standards, and serial dilutions of purified rabbit colonic mucin in 100 μl 0.5 M carbonate buffer (pH 9.6) were coated onto duplicate 96-well polystyrene plates for 2 hours at room temperature. The plates were washed 4 times with phosphate buffered saline containing 0.1% Tween-20 (PBS-T20) using an automated plate washer. The plates were blocked with 3% bovine serum albumin (BSA) in PBS-T20 for 1 hour at 37°C. Following incubation, the plates were again washed, and then incubated with 100 μl of a 1:200 dilution of a 1 mg/ml solution of soybean agglutinin-horseradish peroxidase conjugate (SBA-HRP) in PBS-T20 for 1 hour at 37°C. Following 5 washes with PBS-T20, 85 μl of the substrate 0.4 mg/ml o-phenylene diamine in 0.05 M phosphate-citrate buffer (OPD), was added to each well. After 10 minutes at room temperature, the reaction was stopped with 85 μl of 4 N H_2SO_4. The optical density was read at 490 nm.

Western Blots. Six percent SDS-polyacrylamide gels were prepared (71). Samples, up to 100 μl, were boiled 3 to 5 minutes in 0.25 M 2-mercaptoethanol and 0.1% SDS and applied to gels. Following electrophoresis, gels were either stained for protein with 0.25% Coomassie Blue, or samples were

transferred to nitrocellulose by wet electrophoretic transfer for measurement of lectin-binding activity (mucus) of total glycoprotein.

After transfer, blots to be labelled with lectin were washed with PBS, and blocked in 2.5% casein or 3% BSA in PBS for 1 hour at 37°C. The blots were transferred to a solution of 10 μg/ml SBA-HRP in blocking solution. Following a 1 hour incubation at 37°C, blots were washed 4 times for 5 minutes each in PBS. The substrate solution of 300 mg/ml 4-chloro-1-naphthol was added, and color was allowed to develop for 30 minutes.

Alternatively, transferred blots were stained for total glycoprotein with periodic acid/Schiff (224). Membranes were washed in distilled deionized water for 5 minutes, then incubated in 1% periodic acid, 3% acetic acid for 15 minutes. Following 15 minutes washing with several water changes, modified Schiff's reagent (Fisher Chemical, Pittsburgh, PA) was added, and the membrane was stained for 15 minutes in the dark. The reaction was stopped by a 5 minute incubation in 0.5% sodium bisulfite solution, and the membrane was washed and dried.

Results

Rabbit colonic mucin was successfully purified, as evidenced by a lack of contaminating proteins on SDS-PAGE (figure 4.1, lane 3), and a strong lectin-binding signal in

the stacking gel of a Western blot (figure 4.2, lane 3). No band was seen at 118 kDa, corresponding to the "link" glycopeptide (188), although the mucus was collected with protease inhibitors. Using bovine serum albumin as a standard, the concentration of protein in the purified mucus solution was calculated to be 134 μg/ml by a modified Lowry assay. Using commercially obtained porcine gastric mucin as a standard, the glycoprotein concentration for the same sample was calculated to be 73 μg/ml by the PAS method, but only 17 μg/ml by ELLA.

Coomassie blue staining reveals a major band at 55 kDa in all the cecal filtrates examined (figure 4.1, lanes 5-8), along with a smear that may or may not contain minor bands. This major band most likely represents serum albumin (65). Mucin itself is not visible at these concentrations, in either purified or crude samples. As would be expected, homogenates of ileal and colonic explants demonstrate a large number of protein bands.

For many cecal filtrate and medium/filtrate samples, the only band detectable with soybean agglutinin was in the stacking gel, and consistent with mucus (figure 4.2, lanes 5-7). Other samples showed a smear of high molecular weight material, with a major band at 144 kDa (lanes 8-11). PAS staining of gels (data not shown) gave very similar results to lectin staining. However, only the material in the stacking gel representing mucus was visible in homogenates of ileal and

colonic explants (lanes 12-13), although there was a large number of protein bands (figure 4.1, lanes 9-10).

Discussion

Soybean agglutinin binds to both rabbit colonic mucin and porcine gastric mucin. There appear to be fewer available binding sites on the rabbit mucin, as the amount of purified rabbit colonic mucin calculated from the ELLA was much less than was calculated by more conventional means. This is not surprising, since colonic mucins are more highly sialylated and sulfated than gastric mucins (160), making the internal N-acetylgalactosamine residues less accessible. Therefore, although porcine gastric mucin is used as a standard throughout this study because of availability and consistency, the actual concentrations of rabbit mucus are probably higher than reported. However, the relationships between rabbit samples will remain the same.

It is worth noting that this mucin was collected from a rabbit with experimental mucoid enteropathy. A recent paper (89) demonstrates changes in lectin-binding capacities in mucins from rabbits with mucoid enteropathy. It indicates an increase in binding to soybean agglutinin, as well as other lectins with affinities for internal sugar residues, consistent with the incomplete glycosylation characteristic of many disease states.

The soybean agglutinin is not as specific a marker for mucus as had been expected. However, when non-mucin bands are present in cecal filtrates, they do not appear to change after explant incubation, and can therefore be subtracted out. Furthermore, they are not present in the homogenates of the explants themselves, and so cannot be released into the medium/filtrates following incubation.

Evaluation of Enzyme-Linked Lectin Assay

Introduction

Soybean agglutinin, the lectin used in the Western blots, was also used in an ELLA (27). The mucus-containing sample was used to coat polystyrene wells, and a SBA-HRP conjugate was used to quantitate the amount of mucin bound.

However, a direct assay is generally not considered appropriate for samples that contain only a small concentration of the target molecule. A standard polystyrene ELISA plate binds only 100 ng protein/well (71); the medium/filtrate samples contain approximately 10 mg/ml. Therefore, the majority of an undiluted sample does not bind to the plate, and is removed in the first wash. Only when the sample is diluted will a significant fraction of it bind. Looking at the problem another way, if there is 1 μg/ml mucus in a sample that contains 10 mg/ml total protein, then mucus constitutes 0.01% of that sample. If 100 ng protein binds to

the plate, then only 0.01 ng of mucus will bind, which is at approximately the limit of detection (71).

Three types of modifications were made in an attempt to develop a lectin-based assay that would be more sensitive. One set of modifications was based on classic ELISA techniques (71). Competition (indirect) assays and sandwich assays are used to capture the antigen of interest out of contaminated samples. Crude purification of the samples themselves was also considered, and affinity chromatography and size separation by ultrafiltration were performed. Finally, use of a substrate that has a greater binding capacity (e.g. nitrocellulose vs. polystyrene) allows for greater binding of both contaminants and the molecule of interest, so that it can be accurately quantitated without as great dilution of the sample.

Materials and Methods

Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). The samples used for this experiment were medium/filtrate samples following explant incubation that had been obtained from pilot studies.

Competition assay. Plates were coated with porcine gastric mucin (10 ng/well, 100 ng/well, or 1 μg/well) for 2 hours at room temperature, and then blocked with 2.5% casein for 1 hour at 37°C. 100 μl of serial dilutions of porcine gastric mucin (100 ng/ml stock) or medium/filtrate sample were

added simultaneously with 100 μl of 1, 2, 5, or 10 μg/ml SBA-HRP. Alternatively, mucin containing samples and SBA-HRP were preincubated together for 1 hour at 37°C, and then applied to the plate. Plates were washed with PBS-T20, and 100 μl of the substrate OPD was added. After 10 minutes, the reaction was stopped with 100 μl of 4N H_2SO_4, and the optical density was read at 492 nm.

Sandwich assay. Plates were coated with 2 μg/well of unconjugated soybean agglutinin (*Glycine max* lectin), *Wisteria floribunda* lectin, or *Maclura pomifera* lectin for 2 hours at room temperature, and then blocked with 2.5% casein for 1 hour at 37°C. Then 100 μl of serial dilutions of porcine gastric mucin (100 ng/ml stock) or medium/filtrate sample were added, and incubated 1-2 hours at 37°C. After multiple washes with PBS-T20, 100 μl of 10 μg/ml SBA-HRP was added, and incubated 1 hour at 37°C. Plates were washed with PBS-T20, and 100 μl of the substrate OPD was added. After 10 minutes, the reaction was stopped with 100 μl of 4N H_2SO_4, and the optical density was read at 490 nm.

Centricon filtration. Centricon®-100 (Amicon, Inc., Beverly, MA) filters were rinsed with distilled water, and used according to the manufacturer's instructions to separate 1 ml samples. Both retentates, which should contain only mucus and other molecules > 100,000 Daltons, and filtrates, which should contain molecules < 100,000 Daltons, were assayed in direct ELLA. In addition, these samples were applied to a

6% SDS-polyacrylamide gel to determine the actual protein compositions.

Affinity chromatography. One hundred µl of SBA-sepharose beads were washed and resuspended in 100 µl PBS, and added to 50 µl of sample. After 15 minutes of incubation at room temperature with some mixing, the beads were removed by centrifugation for 1 minute at 1000 x g. The supernatant was retained as "wash 0", an indication of how much mucus did not stick to the beads. The beads were washed 3 times with 1.4 ml PBS. Free N-acetylgalactosamine (200 µl of 10 mM, 0.1 M, 0.5 M, or 1.0 M) was added, and incubated for 15 minutes at room temperature to elute the mucus. Following centrifugation, the eluates were collected and dialyzed overnight vs. PBS. The washes and eluates were analyzed for mucus concentration using a direct ELLA.

Enzyme-linked lectin flow-through assay. An Easy-Titer™ ELIFA apparatus (Pierce, Rockford, IL) was assembled according to the manufacturer's instructions, using either nitrocellulose or a Biodyne® B membrane. The membrane was coated with 100 µl of serial dilutions of porcine gastric mucin (10 µg/ml stock) or medium/filtrate sample, which was pulled through the membrane over a 5 minute period. A 2.5% casein or 3% BSA solution was pulled through over a 10-15 minute period to block the membrane. One hundred µl of 0.1, 0.25, 1, 2.5, or 10 µg/ml SBA-HRP was added, and pulled through in 5 minutes. Plates were washed 3 times with 200 µl

PBS, and 100 μl of the substrate OPD was pulled through as quickly as possible. The reaction was stopped with 100 μl of 4N H_2SO_4, and the optical density was read at 490 nm.

Dot blots. Dot blots were performed using nitrocellulose in a miniblot apparatus. Serial dilutions of mucus or samples in PBS or 0.5 M carbonate buffer, pH 9.6, were used to coat the nitrocellulose for 2-3 hours at room temperature. The blots were removed from the apparatus, washed with PBS, and blocked with 3% BSA for 1 hour at 25°C. Five μg/ml SBA-HRP in PBS was added, and incubated for 1 hour at 25°C. As a control for endogenous peroxidase activity, some blots were not incubated with SBA-HRP, but rather remained in blocking solution. Following four 5 minute washes with PBS, blots were developed by addition of 0.3 g/ml of the substrate 4-chloro-1-naphthol in 50 mM tris, pH 7.6. After 5-10 minutes, the blot was washed with distilled water and dried. Densitometry was performed to quantitate color development.

Results

In all competition assays the optical density reading obtained was a direct reflection of the coating mucus concentration, and SBA-HRP concentration. There was no difference in optical density in the presence or absence of competing mucus or filtrate sample.

In the sandwich assay, the optical density readings depended solely on the type of lectin used for coating the

plate. With soybean agglutinin coating, the optical density ranged from 1.8 to 2.5; for *Wisteria floribunda* the range was 2.1 to 2.7; for *Maclura pomifera* it was 1.5 to 2.3. The presence or absence of mucus had no effect.

Direct ELLA of retentates following Centricon®-100 ultrafiltration did not demonstrate appropriate decreases in optical density with serial dilution. SDS-PAGE demonstrated that the banding pattern for these retentates was identical to that for unfractionated medium/filtrate samples (figure 4.1). No protein was visible by Coomassie blue staining of the ultrafiltrates on SDS-PAGE.

Following affinity chromatography with soybean agglutinin, the material eluted from the beads was calculated to contain approximately 25 ng/ml mucus by direct ELLA. However, the material in "wash 0" (that which did not bind to the beads) contained approximately 250 ng/ml mucus, as measured by ELLA.

With the Easy-Titer™ system, the optical density readings were dependent on the SBA-HRP concentration used for detection, but did not vary with mucus concentration. Background readings (no mucus added) were 2.4-2.8 for 10 μg/ml SBA, 2.1-2.5 for 2.5 μg/ml, 1.9-2.3 for 1 μg/ml, and 1.1-1.4 for 0.25 μg/ml on Biodyne® B membranes. The background values on nitrocellulose were lower; however values for mucus coated wells were also lower, and consequently meaningless.

In contrast, dot blots demonstrated appropriate dilutional effects for both samples and mucus standards; wells which did not contain mucus did not have color development. However, dots containing medium/filtrate samples that were not incubated with SBA-HRP demonstrated significant color development, indicating the presence of endogenous peroxidase activity within the samples. Subtraction of densitometry values obtained in the absence of SBA-HRP from those obtained in the presence of SBA-HRP led to inconsistent data that were deemed unusable.

Discussion

Attempts to increase sensitivity and specificity of mucus quantitation over the direct ELLA described by Cohan (27), and used in the pilot studies were uniformly unsuccessful. Competitive assays for the measurement of mucus have been successful using an antibody-based system (133). However, mucin in solution was not able to compete with bound mucin for lectin binding. It may be that the lectin has a greater affinity for mucin that is bound to the plate as opposed to mucin that is free in solution. Attachment to the plate may allow greater exposure of N-acetylgalactosamine sites to which the lectin may bind. Alternatively, mucin may bind to the lectin in solution, but not block all of the multiple binding sites, thereby allowing the lectin to bind to bound mucus.

Two sets of lectins were used for sandwich assays. At first, the same soybean agglutinin was used to coat the plate and for detection. Soybean agglutinin itself contains N-acetylgalactosamine residues (60), so it is no surprise that once the plate was coated with lectin, the lectin bound maximally, even in the absence of mucin. The other lectins used for coating were *Wisteria floribunda* lectin, a glycoprotein which does not contain N-acetylgalactosamine, and has an affinity for N-acetylglucosamine; and *Maclura pomifera* lectin, a non-glycoprotein that has an affinity for N-acetylgalactosamine (60). Unfortunately, the lectin used for detection was again soybean agglutinin; so that each coating lectin had a direct affinity for the detecting lectin. Use of a different lectin for detection was not attempted, although it might have been successful.

Ultrafiltration was performed to remove contaminants with a molecular weight of < 100,000 Daltons. However, the acrylamide gel demonstrates that essentially all proteins were retained by the filter. Therefore, the contamination in the sample was not reduced, and ultrafiltration was of little benefit. It has been recently reported that nuclease treatment and acidification are required to separate extraneous proteins from mucin (157).

Affinity chromatography was successful in removing contaminants from the final sample. Unfortunately, more mucus was present in the initial wash than in the sample eluted from

the beads. This lack of affinity between the lectin and mucin in solution may relate to the problems with the competition assay.

Nitrocellulose was selected as a substrate with increased binding capacity for the enzyme-linked lectin flow-through assays and dot blots. This worked well for samples containing just mucus, or even samples that had been "spiked" with a known contaminant, such as BSA. Unfortunately, cecal contents contain significant endogenous peroxidase activity. Peroxidase was selected as an enzyme over alkaline phosphatase, which is known to have significant endogenous activity in the gut (71). In the polystyrene plate assay, small amounts of endogenous peroxidase activity can be detected, but these values are very small compared to the mucus/lectin-associated activity. However, when measured by densitometry, nitrocellulose blots incubated without lectin/horseradish peroxidase conjugate often showed greater activity than those incubated with the conjugate. It is not clear why the endogenous peroxidase should bind with more avidity to nitrocellulose than to polystyrene, but the fact exists that it is not feasible to quantitate mucus bound to nitrocellulose.

Therefore, it was not possible to improve upon the direct ELLA using the above techniques. In order to use the direct assay, it is necessary to perform serial dilutions to ascertain that the values used for analysis are not

artifactually low. It is also desirable to select the lowest dilution (highest concentration) possible to obtain the best signal-to-noise ratio.

Harvesting of Mucus from Explants

Introduction

Mucus is a tenacious substance, and a problem in quantitation is collection of the mucus from the tissue (160). In fact, a specific gastric mucosal mucin receptor has been described in the stomach (211). Different methods were evaluated to see if an improvement could be made over simple washing, without seriously damaging tissue and releasing additional mucus. Since mucus is primarily held in association by noncovalent mechanisms (160), acid and base were added to alter pH. Because mucus contains mostly negative charges, and relies on the presence of calcium, magnesium, and other positive ions to maintain gel-forming properties (67), EDTA was added in an attempt to chelate cations, and disaggregate mucus. Finally, N-acetylcysteine, a known mucolytic which breaks down disulfide bonds (200) was added.

Materials and Methods

Explants were collected with a 6 mm Baker's biopsy punch, and incubated in 0.5 ml Trowell's T8 medium with gentamicin and 0.5 ml of a filtrate of cecal contents at 37°C in

95%O_2/5%CO_2 for 1, 2 or 3 hours. Following incubation, one of the following was added: nothing, acid (10 μl, 50 μl, or 100 μl concentrated HCl), base (50 μl 0.5N NaOH), EDTA (1 mg/ml or 10 mg/ml), or N-acetylcysteine (0.1%). The medium/filtrate solution was then collected with a pasteur pipette, after aspirating and discharging five times. Direct ELLA and Western blots were performed to determine the amount of mucus collected. The explants were saved in 10% formalin, and processed for histologic evaluation.

Results

Only the medium/filtrate solutions collected following addition N-acetylcysteine appeared to contain more mucus than those without any exogenous substances (figure 4.2, lane 7 vs. lane 6). Also, at the concentrations of acid used, tissue damage could be seen grossly. Histologically, the explants that had been incubated for 3 hours and collected with N-acetylcysteine all looked healthy and normal (figure 4.3).

Discussion

N-acetylcysteine was deemed to be the best collection method, as it either allows for collection of more mucus, or perhaps just exposes more N-acetylgalactosamine sites on the mucus that is washed off. One hour incubations were chosen for the explants, since stimulated mucus secretion is apparent within that time for several secretagogues, including

acetylcholine (217), histamine (161), mustard oil (161), cholera toxin (189), prostaglandin E_2 (259), and interleukin-1 (27).

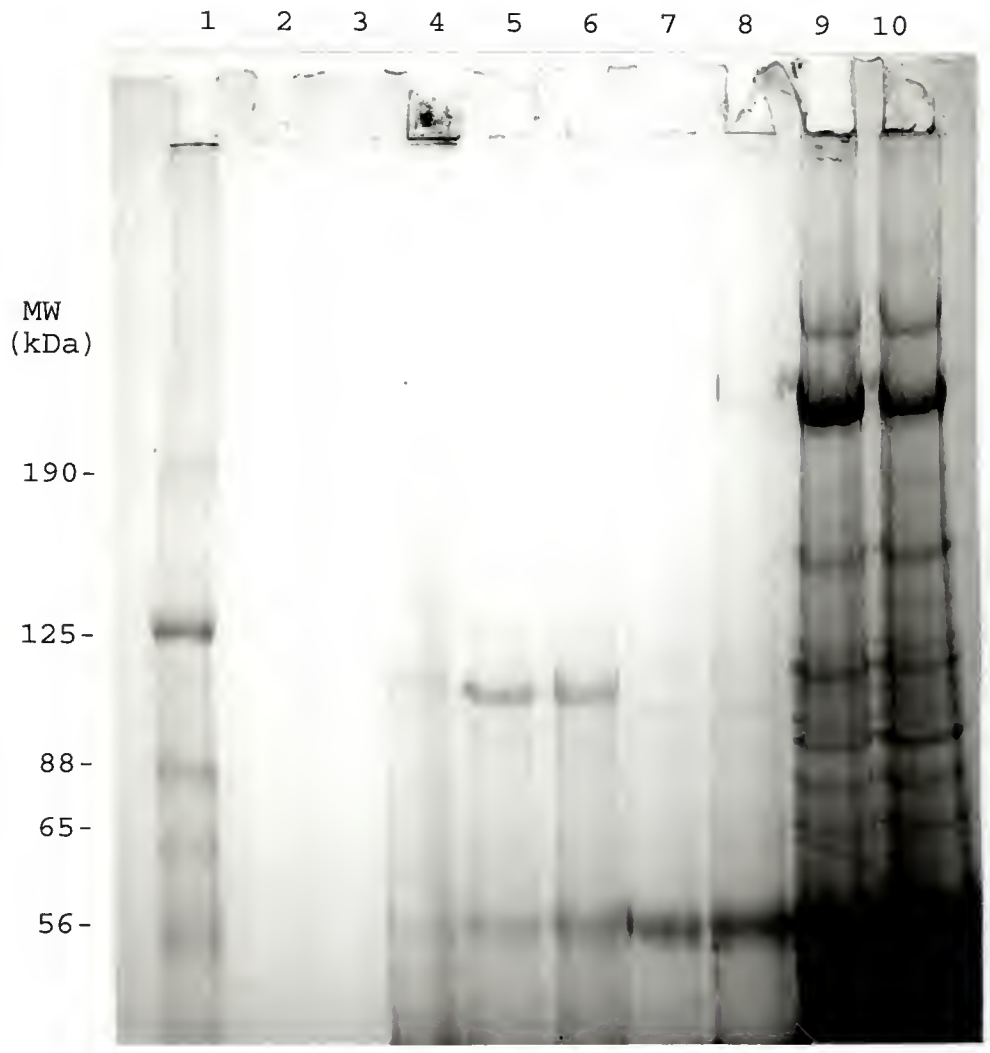

Figure 4.1. 6% SDS-polyacrylamide gel.

Lanes containing: 1) molecular weight standards, 2) porcine gastric mucin, 3) purified rabbit colonic mucin, 4) raw impure rabbit colonic mucus, 5) filtrate from a control rabbit, 6) same filtrate incubated with colon explant, 7) filtrate from a rabbit with experimental ME, 8) same filtrate incubated with colon explant, 9) homogenized ileal explant, and 10) homogenized colonic explant stained for protein with Coomassie blue.

Figure 4.2. Western blot (lectin).

Lanes containing: 1) molecular weight standards, 2) porcine gastric mucin, 3) purified rabbit colonic mucin, 4) raw impure rabbit colonic mucus, 5) filtrate from a rabbit with natural ME, 6) same filtrate incubated with an ileal explant without addition of N-acetylcysteine, 7) same collected with N-acetylcysteine, 8) filtrate from a control rabbit, 9) same filtrate incubated with colon explant, 10) filtrate from a rabbit with experimental ME, 11) same filtrate incubated with colon explant, 12) homogenized ileal explant, and 13) homogenized colonic explant labelled with soybean agglutinin. Since samples were not filtered prior to loading, aggregated material is visible in the wells of lanes loaded with raw mucus or homogenized explants.

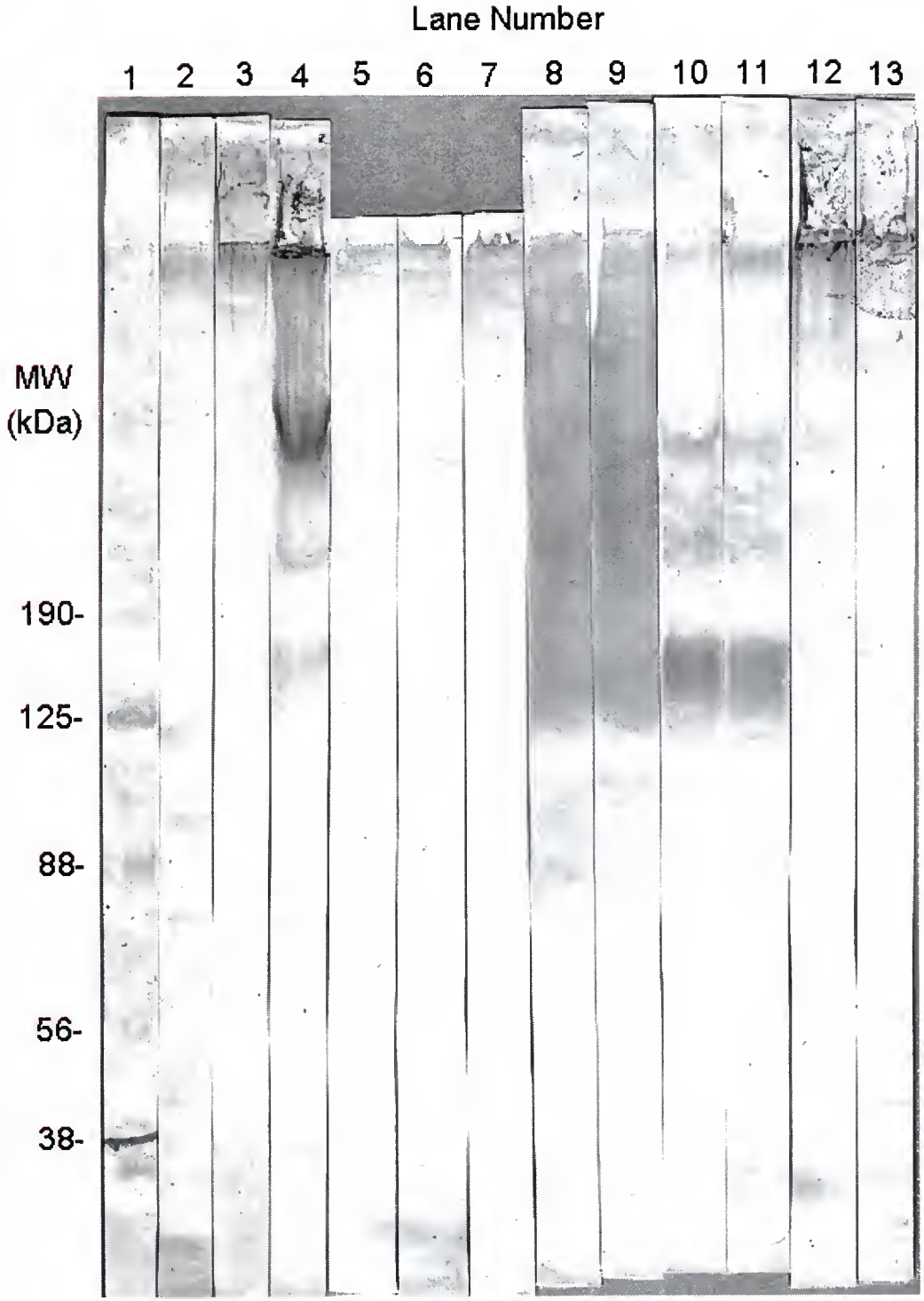
Lane Number
1 2 3 4 5 6 7 8 9 10 11 12 13
MW
(kDa)
190-
125-
88-
56-
38-

Figure 4.3. Explant treated with N-acetylcysteine.

Colonic epithelium is intact, and cells appear viable. Goblet cells containing mucin are visible in the crypts. H&E stain, 475x.

CHAPTER 5
CECAL LIGATION AS A MODEL OF MUCOID ENTEROPATHY

Introduction

Mucoid enteropathy is a serious disease of rabbits, for which the cause is unknown. Research on the disease has been hampered by a lack of good models. Pursuing the hypothesis that mucus hypersecretion is secondary to constipation, Sinkovics (207) was able to induce a mucoid enteropathy-like illness by ligation of the colon. His success rate was approximately 70%. Toofanian and Targowski (229, 235) found that ligation of the cecum, but not cecectomy, also induced a mucoid enteropathy-like syndrome in approximately 70% of rabbits.

In this study specific pathogen-free (SPF, *Pasteurella*-free) rabbits, with no evidence of coccidiosis or other intestinal disease were used. Only 1 out of 8 rabbits developed grossly evident mucus hypersecretion following Toofanian and Targowski's protocol, in which only the cecum was ligated, while vessels and nerves were spared. Therefore, cecal ligations in which the ileocecocolic artery, vein and nerve were incorporated into the ligature were also performed.

Materials and Methods

The experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Nineteen SPF male NZW rabbits, ranging in weight from 1.3 to 2.3 kg, were used in this study. Fecal examinations failed to reveal intestinal coccidia in any rabbit. Five were assigned to be unoperated controls (*group 1*). Eight underwent surgery in which the cecum was ligated without involving major blood vessels or nerves, as described by Toofanian and Targowski (*group 2*). Six underwent surgery in which the distal branch of the ileocecocolic artery, vein, and nerve were incorporated into the cecal ligature (*group 3*). This artery supplies the sacculus rotundus, the second and third limbs of the cecum, and the distal ileum. The branches to the ileum, sacculus rotundus, and third limb of the cecum anastamose with branches of the proximal branch of the ileocecocolic artery (11). For comparison, four rabbits with naturally-occurring mucoid enteropathy also underwent necropsy (*group 4*).

The surgery was performed as follows. Anesthesia was induced with 35 mg/kg ketamine and 5 mg/kg xylazine intramuscularly and was maintained with additional injections of ketamine, or with isoflurane administered by facemask. The rabbits were positioned in dorsal recumbency, and the area surrounding the umbilicus was shaved and prepared for aseptic surgery. A 3-5 cm longitudinal incision was made at the

umbilicus. The base of the cecum was exteriorized. Windows were created through both the ileocecal and cecocolic mesenteries, distal to the sacculus rotundus. Double ligatures of 2-0 silk were passed around the cecum, taking care to either avoid or incorporate the major vessels. Following ligation, ingesta was able to pass freely from the ileum to the colon. The linea alba and then the subcuticular tissues were closed with 3-0 chromic gut in an interrupted pattern.

Clinical observations were made on the rabbits daily. Those with clear mucus in the feces, or that appeared to be in pain, were sacrificed immediately. Those that did not show evidence of a mucoid enteropathy-like syndrome were sacrificed 4 to 10 days following surgery. Body weights were recorded at time of surgery and at necropsy. Prior to euthanasia, rabbits were anesthetized with ketamine/xylazine and blood was collected from the cecal vein. Euthanasia was performed immediately following by an overdose of pentobarbital.

Each rabbit underwent necropsy. Gross mucus hypersecretion was defined as the presence of mucus in the colon that could be lifted with forceps (figure 5.1). Portions of stomach, duodenum, ileum, cecum, proximal colon, distal colon, mesenteric lymph node, kidney, liver, and lung were preserved in 10% neutral buffered formalin. These were processed and sectioned routinely, and slides were stained

with hemotoxylin and eosin. Selected slides were also double stained with periodic acid/Schiff and alcian blue.

Cecal contents were collected and filtrates were prepared for additional studies. The entire cecum was removed at the ligature site and weighed. Fifty ml of sterile 0.9% saline was infused, and the contents were massaged gently. The contents were expressed into a sterile container and the empty cecum was weighed. The volume of cecal contents in saline was recorded. The slurry was centrifuged at 5000 rpm (3000 x g), 4°C for 15 minutes. The supernatant was collected, and centrifuged at 12,000 rpm (15,000 x g), 4°C for 30 minutes; then at 18,000 rpm (30,000 x g) for 1 hour, and then 2 hours. The resulting supernatant was then filtered through a 0.22 μ sterilizing membrane filter with a prefilter. The sterile filtrates and sera from the cecal vein were stored at -70°C until use. 1 ml aliquots were dried overnight at 90°C and weighed.

Results

Surgery was uneventful in all rabbits. One rabbit (group 2) suffered dehiscence of the skin incision the day after surgery. The skin was resutured under ketamine/xylazine anesthesia, and there were no further problems. Food consumption was decreased following surgery, but rabbits continued to drink. All rabbits had a greatly decreased fecal output following surgery. The day after surgery, all but one

of the group 2 rabbits had normal feces intermingled with soft feces or diarrhea. The remaining rabbit passed small, dry pellets. All group 3 rabbits had diarrhea following surgery.

Six group 2 rabbits were sacrificed 4 days after surgery; they had abnormal feces at the time of death. Two group 2 rabbits were sacrificed 10 days following surgery. They both had normal feces from the fifth day following surgery until death. Five group 3 rabbits passed copious amounts of mucus in the feces on the third day following surgery, and were sacrificed. The remaining rabbit did not excrete mucus, but continued to have diarrhea, and was sacrificed on day 5. Group one rabbits remained healthy throughout the study.

Table 5-1. Results of cecal ligation.

Group	Treatment	Number of Animals	Mucus Secretion	Cecal Necrosis
1	Control	5	0	0
2	Ligation/ without vessels	8	1	0
3	Ligation incorporating vessels	6	6	5 (3 seen grossly)
4	Natural disease	4	4	0

At necropsy (see table), all group 3 and 4 rabbits had gross evidence of mucus hypersecretion in the colon (figure 5.1). One group 2 rabbit had evidence of mucus hypersecretion in the colon, while one had white chunks of inspissated mucus in the cecum. Using a z-statistic to compare binomial

proportions, the difference in the percentage of rabbits demonstrating mucus hypersecretion is statistically significant ($P < 0.003$). In three out of 6 group 3 rabbits, areas of the cecum (2 to 5 cm in diameter) appeared necrotic grossly (figure 5.2), and there were adhesions of the cecum to other organs in 4 of these rabbits. In one rabbit, the cecal ligature was no longer present. This rabbit did not excrete mucus antemortem, but mucus was present in the colon. These abnormalities were not seen in group 1, 2 or 4 rabbits.

All rabbits that underwent surgery lost weight (figure 5.3; Appendix B), while control rabbits gained weight. Group 3 rabbits lost more weight than group 2 rabbits ($P < 0.05$).

The major histologic abnormality was segmental severe full-thickness cecal necrosis with acute inflammation, edema, and hemorrhage of the adjacent tissue in five out of six group 3 rabbits (figure 5.4). In one rabbit, the mucosa of a cecal fold was necrotic, while surrounding tissue was normal. In another, a cecal fold was spared while adjacent tissue was necrotic. In the two group 3 rabbits without severe necrosis there was hemorrhage and edema in the mucosal layer. One of these also had acute peritonitis. No group 2 rabbit had full thickness necrosis, but four out of eight had mild to moderate peritonitis, and two had hemorrhages in the lamina propria. These changes were apparently related to surgical technique.

In the sections taken from rabbits with the naturally-occurring disease (group 4), two rabbits had mild acute

inflammation of the cecal mucosa. Several stages of coccidia and a few pinworms were present in one of these rabbits with spontaneous ME. One rabbit in each of the four groups appeared to have goblet cell hyperplasia (figure 5.5).

In the proximal colon, two group 2 rabbits, and two rabbits with naturally-occurring disease demonstrated acute mucosal inflammation. One group 3 rabbit had acute peritonitis. Goblet cells appeared depleted of acidic (alcian blue-staining) mucin in four group 3 rabbits, and in one section from a group 4 rabbit (figure 5.6). In the distal colon, four out of eight group 2 rabbits, four out of six group 3 rabbits, and all four group 4 rabbits demonstrated mucosal inflammation (figure 5.7). This was mild in most animals, but was more severe than in any control animal. This was usually characterized by heterophils, but in a few rabbits plasma cells were predominant. Two group 2 rabbits, and one rabbit each in group 3 and 4 had necrosis of the surface epithelium with focal erosions.

In the small intestine, mild inflammatory infiltrates were seen sporadically in all groups. Dilated lacteals were seen in the one group 2 rabbit that secreted mucus, in three group 3 rabbits, and in one group 4 rabbit. Villous atrophy was present in the ileum of one group 3 rabbit. Villi appeared shorter overall in group 3 rabbits, but quantitative measurement was not attempted. Minimal goblet cell hyperplasia was present in one rabbit each in groups 2, 3, and

4. There were no abnormal findings in the stomach of any rabbit.

There were no significant findings in the lungs. The mesenteric lymph nodes appeared congested in two group 3 rabbits. Hepatic congestion was seen in two group 3 rabbits. These tissues had not been saved from group 4 rabbits.

Although the ratio of empty cecal weights relative to body weights were similar for the three groups (figure 5.8), the full cecal weights relative to body weight were significantly higher in the control rabbits. This corresponds to the finding that the volume and weight of cecal contents were significantly decreased in group 2 and 3 rabbits. Other parameters, such as the volume of filtrate collected relative to the volume of cecal contents processed and the dry weight of the final cecal filtrate, were not significantly different between groups (Appendix B).

Discussion

Toofanian and coworkers (229, 233, 234, 235, 236) reported a 70% incidence of mucoid enteropathy-like syndrome following cecal ligation without incorporation of vessels or nerves. The success rate in this study was substantially lower (1 out of 8, or 12.5%). One possible explanation for this is the quality of the rabbits used. No mention is made of the status of the rabbits used in their experiments, therefore it is likely that conventional rabbits were used.

On the other hand, we used SPF rabbits, which were free of intestinal coccidia. Coccidia are known to increase mucus secretion (114), and it is possible that their presence has a synergistic effect with cecal ligation, resulting in a higher incidence of mucus hypersecretion.

Incorporation of the ileocecocolic vessels into the cecal ligature results in a more reproducible model of mucoid enteropathy in the rabbit. It does have drawbacks, however. Severe cecal necrosis was seen with this model, but has not been reported in association with the natural disease (142, 238, 251). On the other hand, mild cecal necrosis has been reported (238, 251).

Histologically, there were many similarities between ligation-induced mucoid enteropathy and the natural disease. In both cases inflammatory changes were inconsistent, but multifocal mild infiltrates of heterophils and mononuclear cells were seen throughout the intestinal tracts of some animals (142, 229, 235, 238, 251). Goblet cells were depleted of acidic mucus in the colon (238). Edema of intestinal tissues or mild nephrosis was an occasional finding (142, 238).

In this study the consistent goblet cell hyperplasia of the small intestine that is supposed to be characteristic of mucoid enteropathy (142, 229, 238, 251) was not observed. However, the number of rabbits with goblet cell hyperplasia was similar, whether or not the disease was natural or

experimentally induced. Cecal ligation may induce villous atrophy in the ileum (229, 235), presumably due to small intestinal bacterial overgrowth; this finding has not been reported in the natural disease.

Although the cecum maintained its size in relation to the weight of the animal following cecal ligation, the amount of cecal contents, both weight and volume, was decreased. The dry weights for the final cecal filtrates were not significantly different for the three groups. Since the same volume of saline (50 ml) was added to each cecum for the collection of cecal contents, the cecal contents from rabbits in Groups 2 and 3 were diluted more than the controls, and therefore must have started out more concentrated. This is consistent with reports that cecal contents from rabbits with mucoid enteropathy are drier than normal (207, 238).

In two additional adult rabbits, only the ileocecocolic vessels and nerve, but not the cecum, were ligated (data not shown). Neither rabbit showed any sign of illness, or exhibited any gross abnormalities at necropsy. Therefore, it appears that there are two factors necessary for induction of experimental mucoid enteropathy in SPF rabbits: 1) stasis of cecal contents induced by cecal ligation, and 2) interference with the blood and/or nerve supply.

Cecal ligation has also been performed on rabbits as part of the reversible ileal tie adult rabbit diarrhea ("RITARD") model for the study of cholera and enterotoxigenic E. coli

(222). No diarrhea or illness was seen following cecal ligation and temporary ileal occlusion in the absence of pathogenic organisms. However, mucoid diarrhea was seen when 10^{10} Vibrio cholerae organisms were inoculated into the jejunum concurrent with cecal ligation, as opposed to the watery diarrhea that was seen when organisms were inoculated in conjunction with ileal occlusion. This supports the finding in this study that an additional insult is required along with cecal ligation to stimulate mucus hypersecretion.

Figure 5.1. Excessive colonic mucus.

Single-banded proximal colon from group 3 rabbit #A7, opened to reveal mucus.

Figure 5.2. Gross cecal necrosis.

Cecum from group 3 rabbit #A7. The base of the cecum is severely congested. The necrotic area in the body of the cecum ruptured during removal from the carcass.

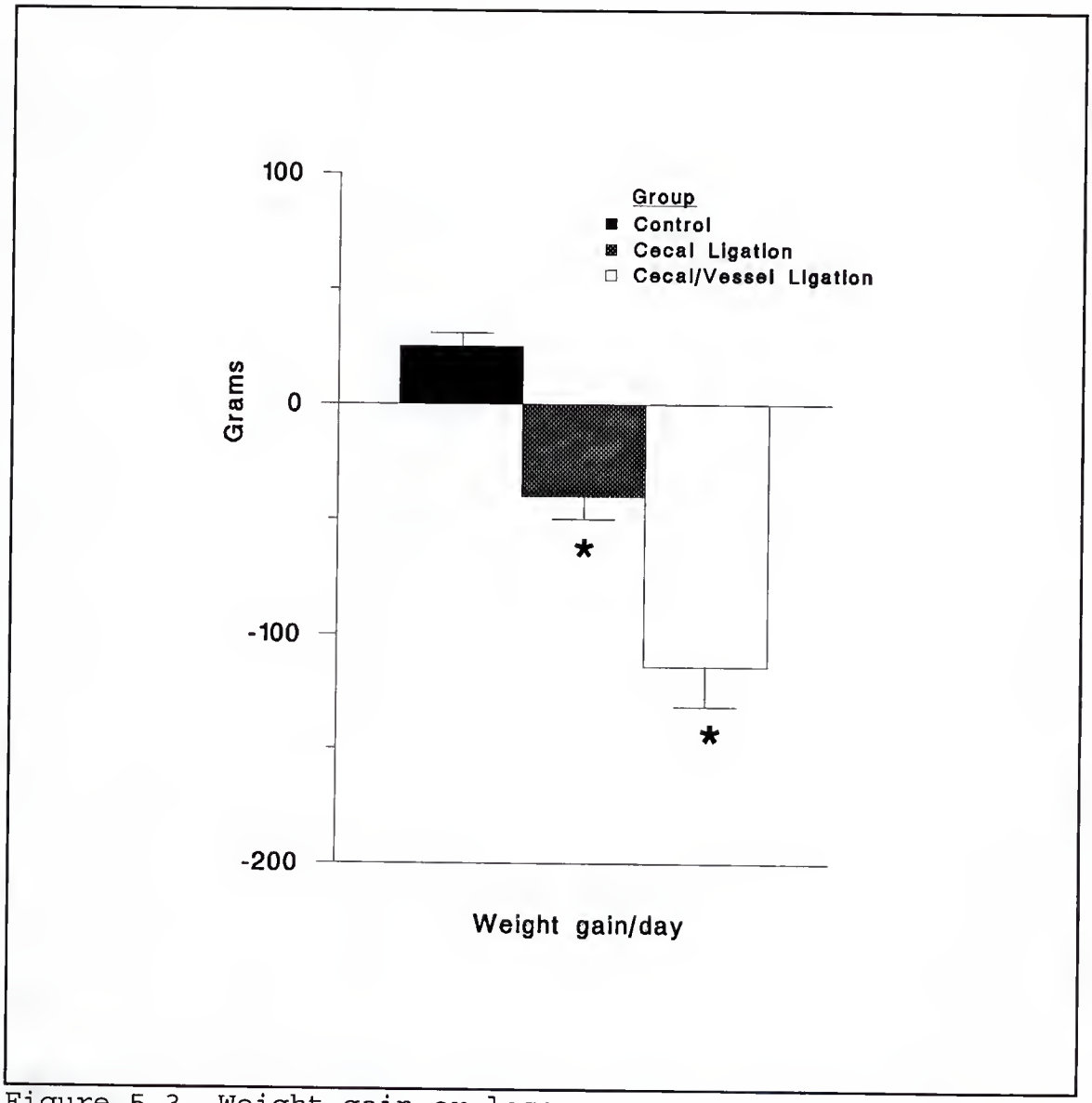

Figure 5.3. Weight gain or loss.

Weight gain or loss per day following cecal ligation surgery, in grams. Bars represent mean + SEM. N = 5 (control), 8 (cecal ligation), or 6 (cecal/vessel ligation). * = significantly different from control ($P < 0.05$).

Figure 5.4. Cecal necrosis and inflammation.

At the left is relatively normal cecal tissue, however at the right there is full thickness necrosis with a mixed inflammatory infiltrate. H&E stain, 45x.

Figure 5.5. Goblet cell hyperplasia.

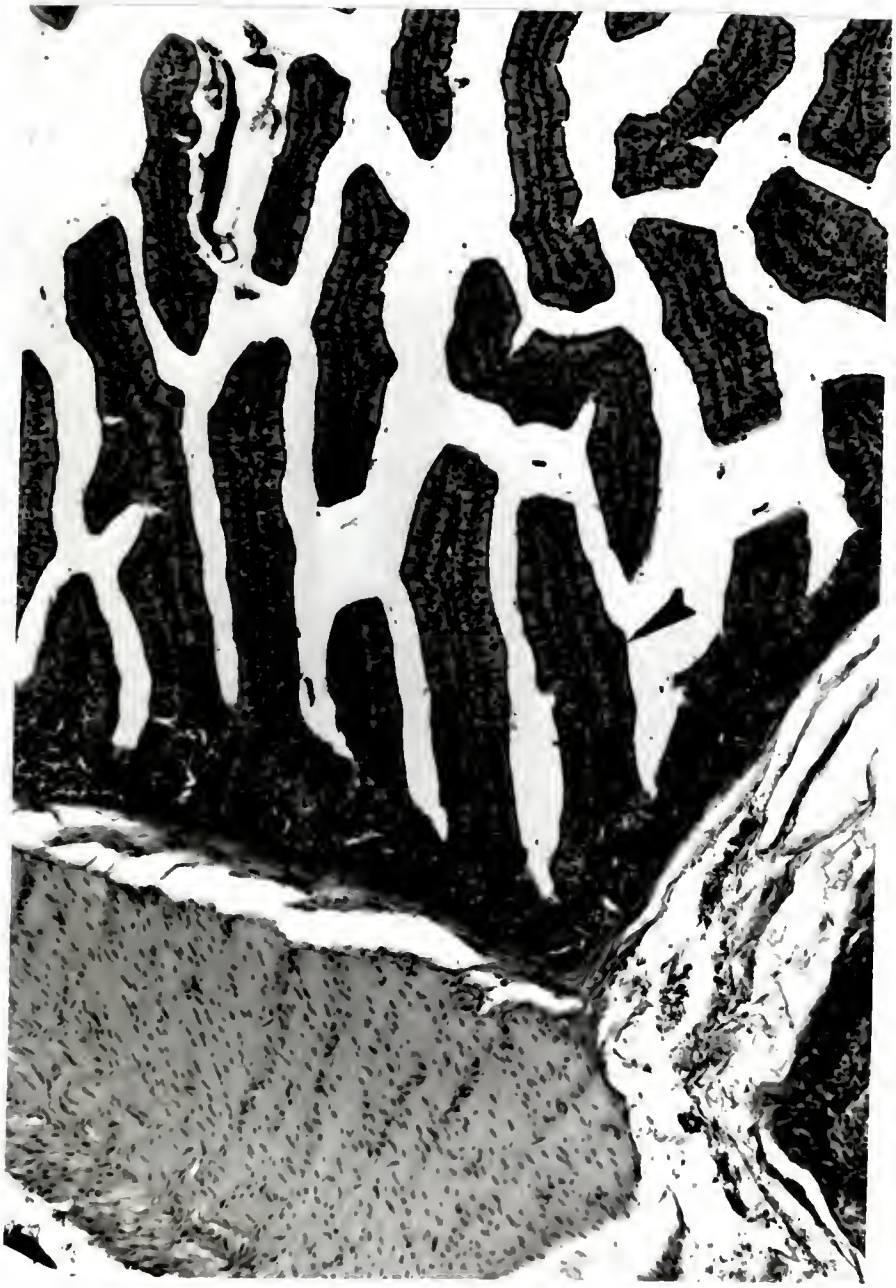

Figure 5.5a. Normal jejunum, stained for mucin with PAS/alcian blue. Most goblet cells contain neutral mucin, staining with PAS (arrow). 115x.

Figure 5.5b. Jejunum from rabbit with experimental mucoid enteropathy, stained with PAS/alcian blue. Numerous goblet cells contain acidic mucin, staining with alcian blue (arrows). 115x.

Figure 5.6. Depletion of acidic mucin.

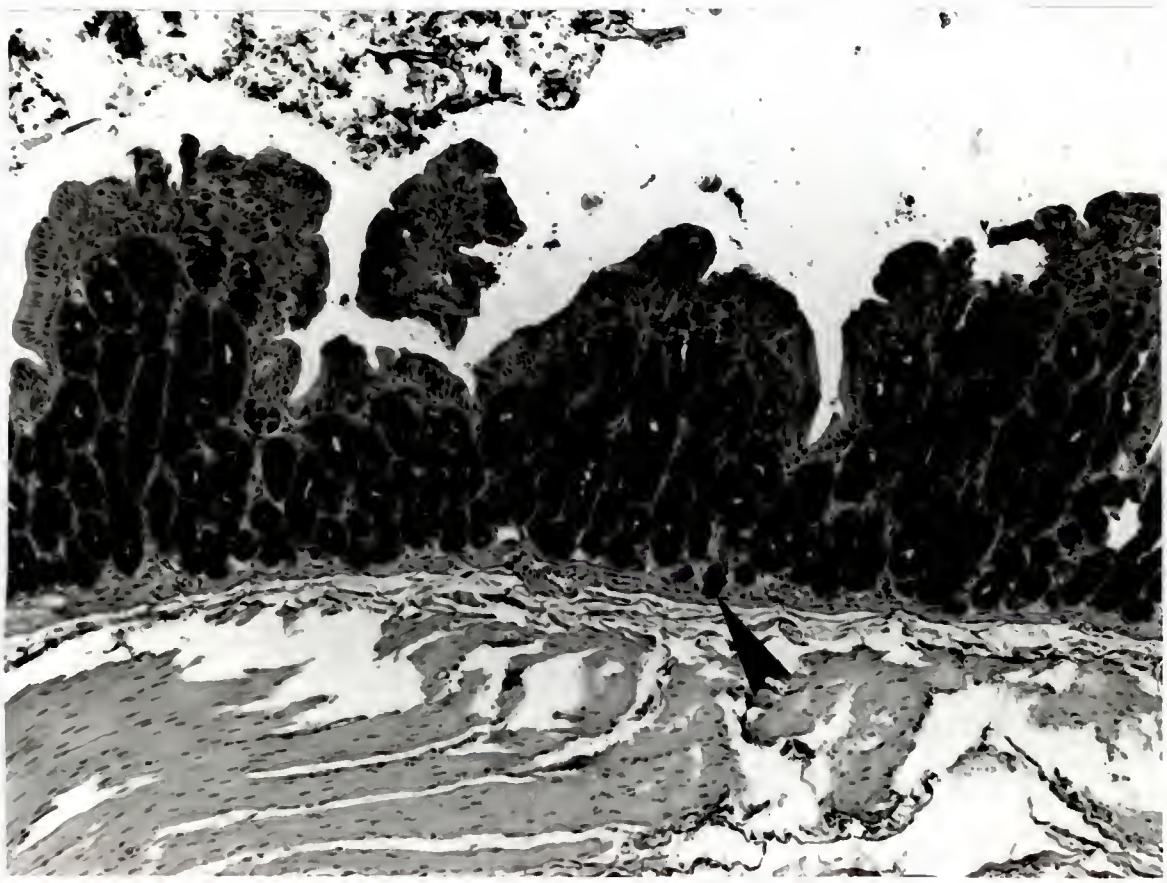

Figure 5.6a. Normal distal colon, stained for mucin with PAS/alcian blue. Mucin-containing goblet cells are prominent throughout the mucosa. Neutral (PAS-stained) mucins are generally confined to deep in the crypts (arrow). 115x.

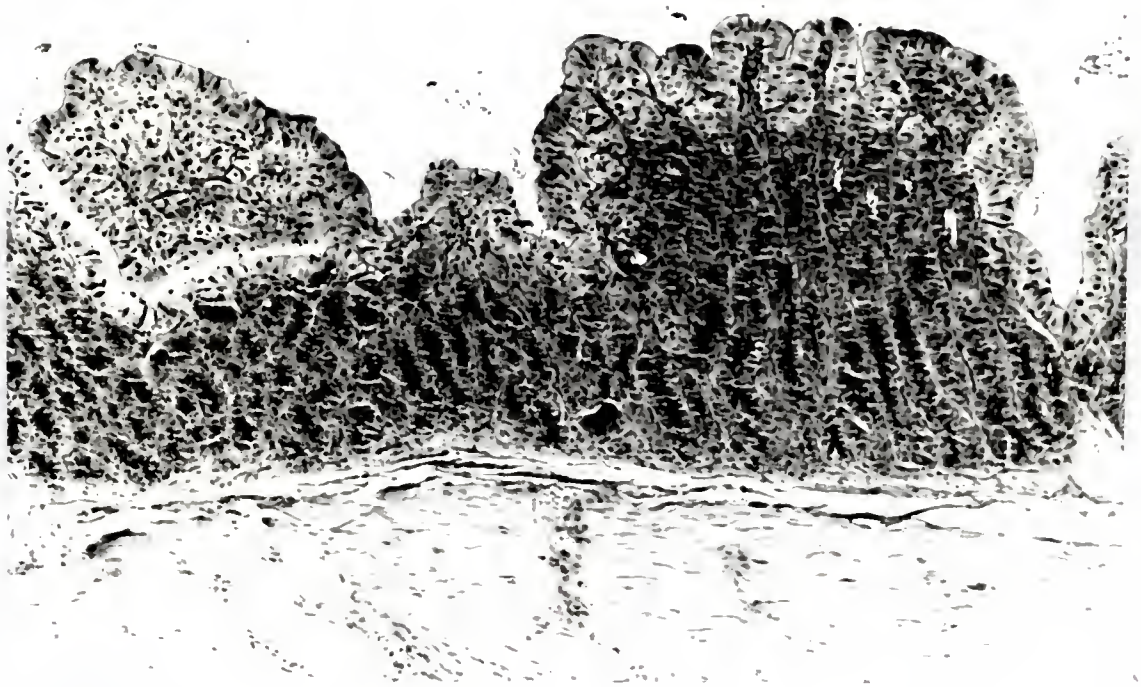

Figure 5.6b. Colon from a rabbit with experimental mucoid enteropathy. Goblet cells contain little mucus, particularly in the middle portion of the crypts. PAS/alcian blue stain, 115x.

Figure 5.7. Colonic inflammation.

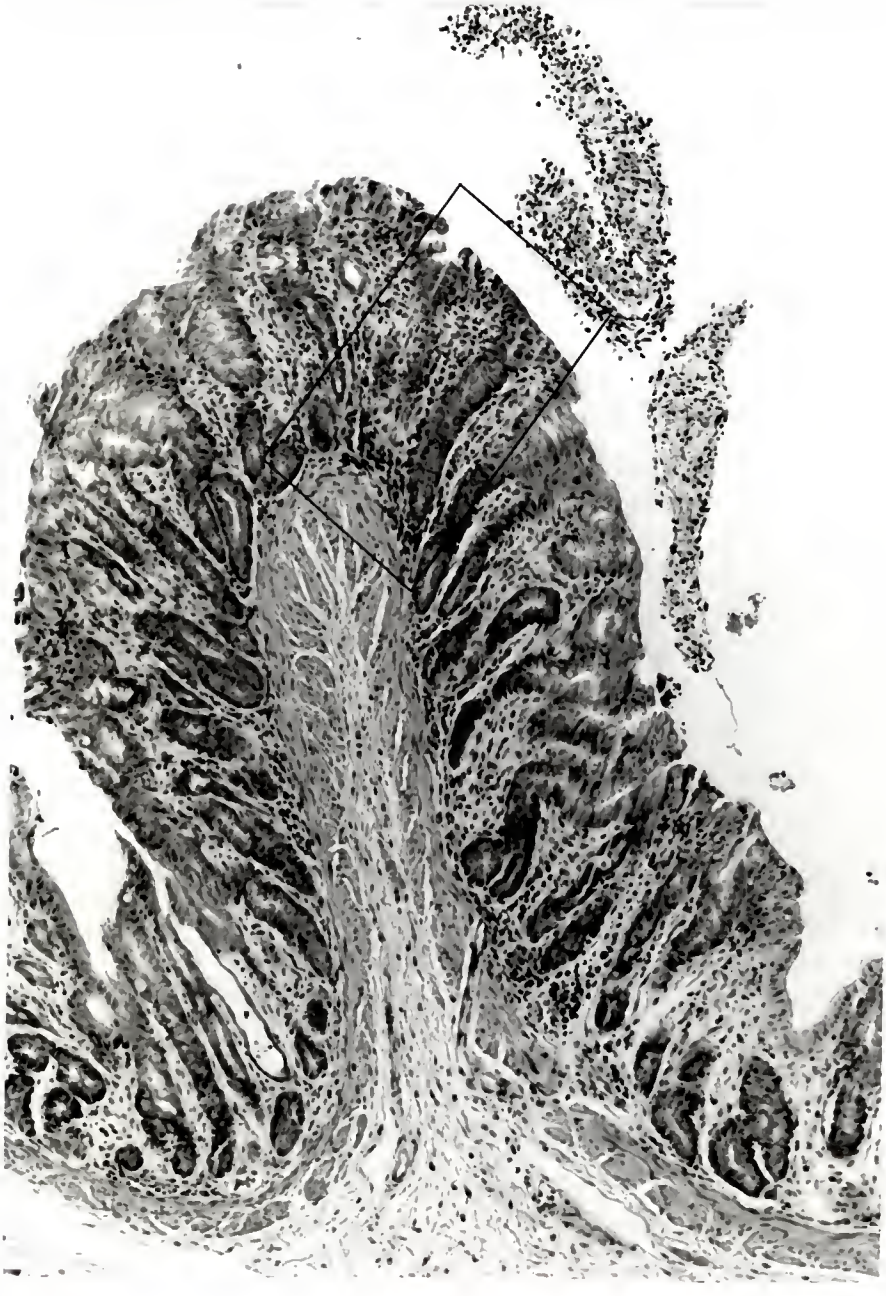

Figure 5.7a. Distal colon from a group 2 rabbit. There is loss of epithelium, and exudation of purulent material into the intestinal lumen. Box indicates area shown in figure 5.7b. H&E stain, 115x.

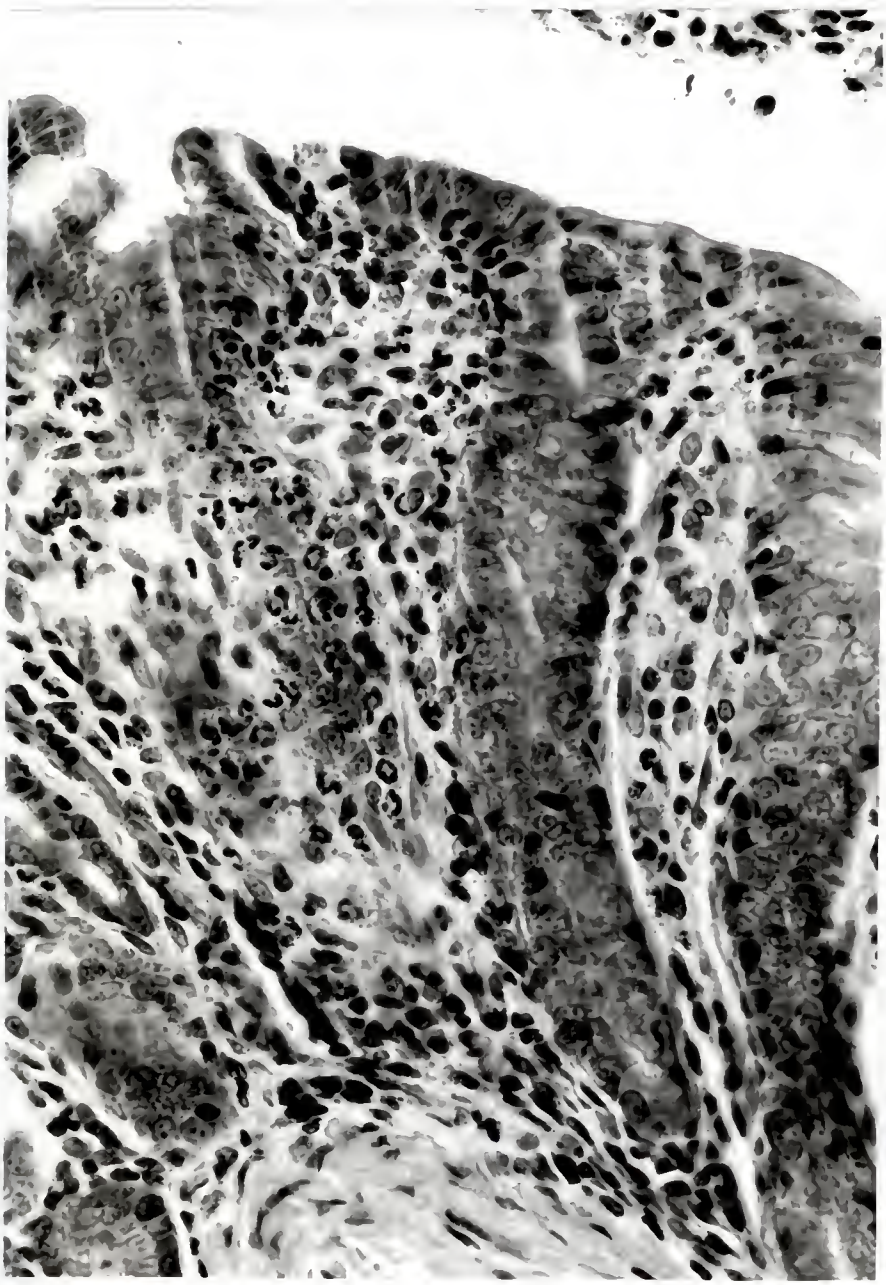

Figure 5.7b. Higher magnification of colon in 5.7a. Epithelial cells are flattened and numerous heterophils are present in the lamina propria. H&E stain, 450x.

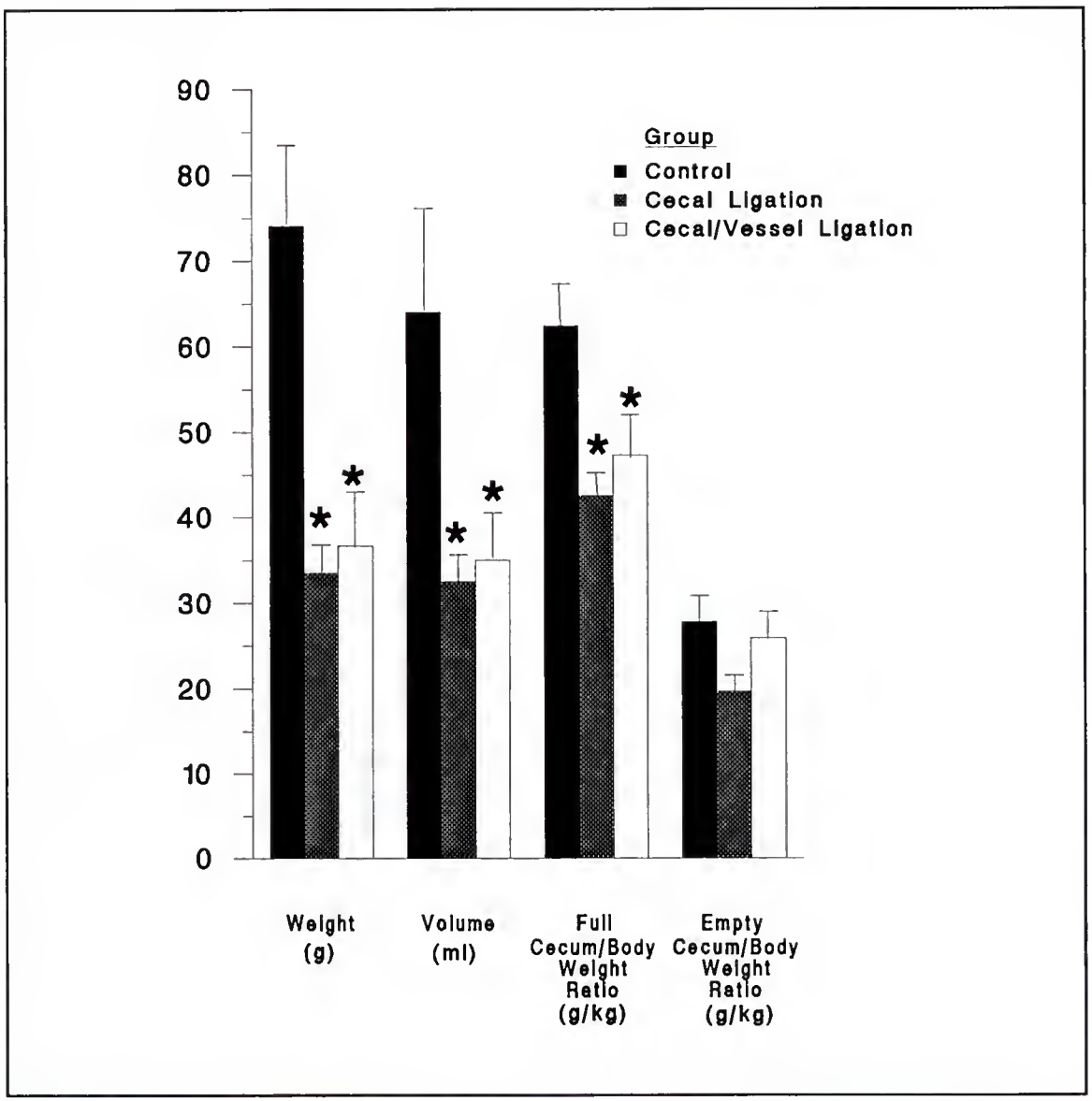

Figure 5.8. Characteristic of cecal contents.

Weight and volume of cecal contents collected, and the ratio of full and empty cecal weights (in grams) to body weights (in kg). Bars represent mean + SEM. N = 5 (control), 8 (cecal ligation), or 6 (cecal/vessel ligation). * = significantly different from control ($P < 0.05$).

CHAPTER 6
MUCUS SECRETION FROM INTESTINAL EXPLANTS

Introduction

It has been hypothesized that there is a substance in the cecal contents of rabbits with experimental ME that causes goblet cell hyperplasia (236). Pilot studies (chapter 3) suggested that cecal contents from a rabbit with naturally-occurring mucoid enteropathy stimulate mucus secretion from intestinal explants. This study tests the hypothesis that there is a mucus secretagogue within the luminal cecal contents of rabbits with cecal ligation-induced mucoid enteropathy.

Materials and Methods

Cecal contents were collected from control and experimental rabbits, as described in chapter 5. Filtrates from all 5 control rabbits (group 1) were used for this study. Eight rabbits had undergone cecal ligation without ligation of the ileocecocolic artery (group 2); however minor vessels had been ligated in three of these rabbits. Such rabbits were excluded, leaving 5 rabbits in group 2. In one of the 6 rabbits that had undergone cecal and vessel ligation (group 3), the ligature was not present at necropsy; cecal contents

from this rabbit were not used, leaving 5 rabbits in this group also.

Five male SPF NZW rabbits, weighing 1.8 to 2.3 kg were used as explant donors. The rabbits were sedated with 35 mg/kg ketamine, and euthanasia was performed with pentobarbital. The ileum and single-banded (aboral) portion of each proximal colon was removed, and opened along the mesenteric border. These intestinal sections were rinsed to remove digesta, first in sterile 0.9% saline, and then in HBSS containing 100 μg/ml gentamicin.

Fifteen explants were collected from each site in each rabbit, using a 6 mm Baker's biopsy punch. To minimize the effects of variation in mucus-secreting capacity along the length of the gut, explants were collected in groups of 3; one for each treatment group from comparable locations along the length of the intestine. For each explant donor rabbit, the filtrates from the three groups were matched in different combinations. The explants were placed in wells containing 0.5 ml Trowell's T8 medium with 100 μg/ml gentamicin and 0.5 ml of one of the cecal filtrates. The explants were incubated 1 hour at 37°C in 95% O_2/5% CO_2. Additional wells containing each medium/filtrate solution without explants, "blanks", were also incubated.

Following incubation, 20 μl of 5% N-acetylcysteine (final concentration 0.1%) was added to each sample, and the medium/filtrate solution was collected. The samples were

frozen at -70°C for enzyme-linked lectin assay (ELLA), and the explants were weighed.

ELLA analyses were performed by modification of a previously described technique (27). Porcine gastric mucin standards, and 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280 dilutions of medium/filtrate samples in 100 μl 0.5 M carbonate buffer (pH 9.6) were coated onto duplicate 96-well polystyrene plates for 2 hours at room temperature. An additional plate was coated in the same way to be used as an endogenous peroxidase control plate. The plates were washed 4 times with PBS-T20 using an automated plate washer. The plates were blocked with 3% BSA in PBS-T20 for 1 hour at 37°C. Following incubation, the test plates were again washed, and then incubated with 100 μl of a 1:200 dilution of a 1 mg/ml solution of SBA-HRP in PBS-T20 for 1 hour at 37°C. The endogenous peroxidase control plate remained in the blocking solution. Following 5 washes with PBS-T20, 85 μl of the substrate OPD was added to each well. After 10 minutes at room temperature, the reaction was stopped with 85 μl of 4 N H_2SO_4. The optical density was read at 490 nm.

Each sample was corrected for endogenous peroxidase activity by subtracting the corresponding values on the control plate (maximum = .032 optical density units). A standard curve was prepared for each plate, and the concentration of mucus in the undiluted sample (in relation to porcine gastric mucus), was calculated for each well. The

1:80 dilution was selected for further analysis, as not having artifactually low readings due to interference by contaminating proteins, or too much variability. The amount of mucus in the medium/filtrate in the absence of an explant ("blank") was subtracted from each sample to give the amount of mucus secreted from the explant.

All results were subjected to analysis of variance using a randomized block design. When significant main effects were found, a Fisher's Least Significant Difference test was used to determine differences between groups.

Results

In the absence of any explant the control medium/filtrate mixtures contained more mucus than those of either of the surgically ligated groups (7.09 μg/ml for group 1 vs 0.32 μg/ml for group 2 and 0.47 μg/ml for group 3; figure 6.1, Appendix C). When the outlier (rabbit A2) is removed from the calculations, the mucus concentration for group 1 (1.31 μg/ml) is significantly greater than for the ligated groups ($P < 0.05$). There was a great deal of variation in mucus concentration due to the explant source following incubation with ileal explants, but no consistent differences due to filtrates were seen (Appendix C). On the other hand, there was little evidence of secretion from colonic explants incubated with control filtrates, while explants from different rabbits were consistent in showing an increase

following incubation with filtrates from cecal-ligated rabbits.

Significantly ($P < 0.05$) more mucus was secreted from colonic explants when incubated with cecal filtrates from rabbits that underwent cecal ligation than when incubated with filtrates from control rabbits (figure 6.2). This effect was more striking in the rabbits where only the cecum and not the vessel was ligated. Most of this was due to the filtrate from a single rabbit (A5), which had grossly visible mucus present in the colon at necropsy. There was no difference between groups in secretion from ileal explants.

Discussion

Cecal filtrates from control rabbits contain more mucus than those from rabbits with experimentally-induced mucoid enteropathy. At first this seems paradoxical, since mucus was visible in the ceca of some of these rabbits. However, when mucus is in high enough concentration locally (≥25 mg/ml, 51), it will form an insoluble polymeric gel which is unable to pass through a .22 μm filter, and is therefore removed from the final filtrate. Alternatively, control filtrates may contain a non-mucus substance that contains N-acetylgalactosamine residues.

Once the baseline amount of mucus present in the filtrate initially is subtracted, the results may be analyzed more easily. No secretagogue effect is seen in the ileum in this

experiment. A major confounding factor was the wide variation in measured mucus depending on the source of the explant. This may be a real phenomenon, reflecting variation in ileal mucus secretion based on individual factors such as vagal tone, circadian rhythms, or the timing of the last meal (47, 73, 98). However, another plausible explanation is that this is a washing artifact. In the colon, there are no villi, so removal of endogenous mucus from its surface (although not from the crypts) should be more easily accomplished. Therefore, the individual variations seen here in the amount of mucus released from the ileal explants may just reflect the difference in the amount of mucus trapped in the villi following washing, which was then released into the medium/filtrate during incubation.

The fact that filtrates from certain rabbits induced mucus secretion more strongly than others could result from the production of the secretagogue by microbial flora, which can vary a great deal between individual rabbits; as opposed to the secratagogue being a host product, such as an inflammatory mediator, which one would expect to see in all rabbits.

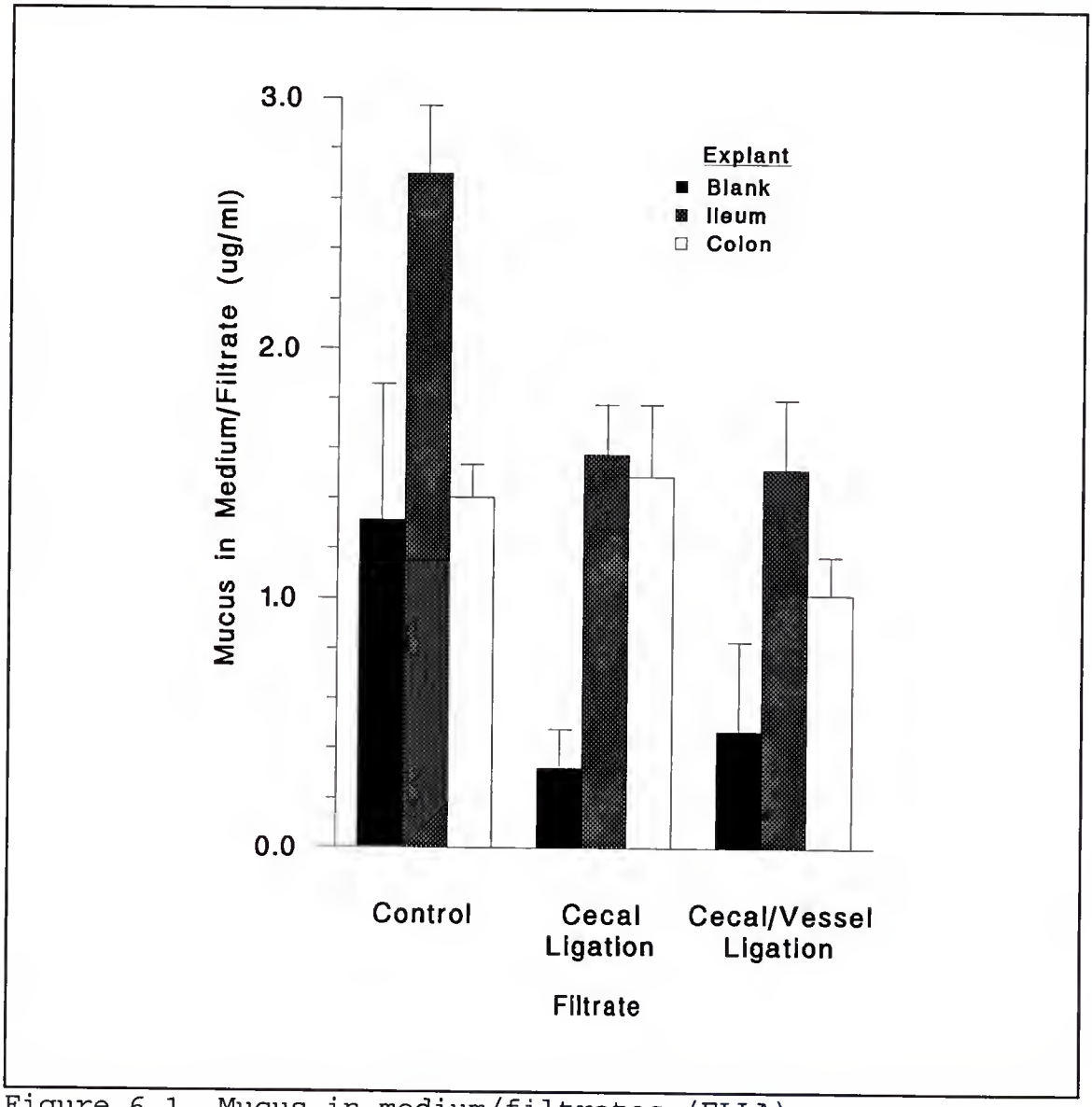

Figure 6.1. Mucus in medium/filtrates (ELLA).

Calculated mucus (porcine gastric mucin equivalents) in medium/filtrates, as measured by ELLA. Bars represent mean + SEM. N=5 (blanks) or 25 (with explants).

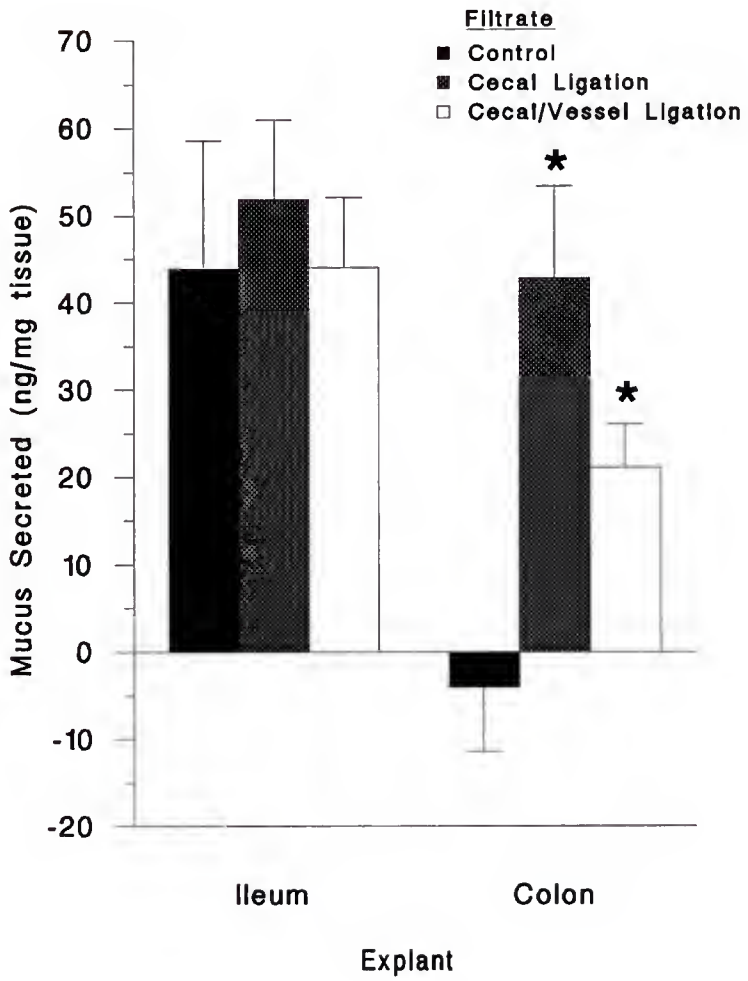

Figure 6.2. Mucus secretion from explants.

Mucus secretion determined by subtracting the amount of mucus in the medium/filtrate incubated without explants (as measured by ELLA) from the amount incubated with explants. Values shown are relative to the wet weight of the explant. Bars represent mean + SEM. N = 25. * = significantly different from control ($P < 0.05$).

CHAPTER 7
COMPARISON OF ELLA AND *IN VITRO* LABELLING

Introduction

Since the preceding data support, but do not prove, the hypothesis that there is mucus secretagogue activity in the luminal cecal contents of rabbits with experimentally-induced mucoid enteropathy, further work was performed. In this chapter two independent methods were used to measure mucus secretion from intestinal explants; ELLA, and secretion of mucus that had been radiolabelled in vitro. Two filtrates from each group: control, cecal ligated, and cecal ligated with gross mucus hypersecretion (including a rabbit that did not undergo vessel ligation), were selected to form sample pools to maximize the differences between the groups.

Materials and Methods

One male 2.0 kg SPF NZW rabbit was used. The rabbit was sedated with 35 mg/kg ketamine, and euthanasia was performed with pentobarbital. The single-banded (aboral) portion of the proximal colon was removed, and opened along the mesenteric border. This intestinal section was rinsed to remove digesta, first in sterile 0.9% saline, and then in HBSS containing 100 μg/ml gentamicin.

Forty explants were collected, using a 6 mm Baker's biopsy punch. To minimize the effects of variation in mucus-secreting capacity along the length of the gut, explants were collected in groups of 4; one for each treatment group taken from comparable locations along the length of the intestine. Each explant was placed in a polystyrene well containing 1 ml Trowell's T8 medium with 100 μg/ml gentamicin. Half of these wells also contained 2 μCi ^{3}H-glucosamine. The explants were incubated for 2 hours (pulse) at 37°C in 95% O_2/5% CO_2.

Following this incubation, the explants were washed by transferring them to individual wells containing 1 ml HBSS, and the wash buffer was replaced 3 times. The explants were then transferred to wells containing 0.5 ml Trowell's T8 medium with 100 μg/ml gentamicin and 0.5 ml of one of the following: control pool cecal filtrate (rabbits A1 and A12); control pool cecal filtrate (rabbits A1 and A12) + 4 μg/ml bethanechol (final concentration = 10^{-6}M); ligation/no mucus pool (rabbits A6 and H2); or ligation/mucus pool (rabbits A5 and H7). Note that rabbit A5 underwent cecal ligation without vessel ligation; however mucus hypersecretion was present at necropsy, and filtrates from this rabbit were the most potent stimulators of mucus secretion from explants. The explants were incubated 1 hour at 37°C in 95% O_2/5% CO_2. Additional wells containing medium/filtrate solutions without explants, "blanks", were also incubated.

Following incubation, 20 μl of 5% N-acetylcysteine (final concentration 0.1%) were added to each sample, and the medium/filtrate solution was collected. The non-radioactive samples were frozen at -70°C for enzyme-linked lectin assay (ELLA).

Radioactive medium/filtrate samples and radioactive explants were transferred to scintillation vials. One ml water was added to each radioactive explant. Then, prior to counting, 3.5 ml scintillation fluid (Scintiverse BD, Fisher Scientific, Pittsburgh, PA) was added to each of the radioactive medium/filtrate solutions and the radioactive explants. Radioactive standards containing 0, 0.5, 1, and 2 μCi of ^{3}H-glucosamine in Trowell's T8 medium were also counted.

Samples were counted on day 1, and recounted on day 4 for verification. Counts were corrected for quenching using an external standard ratio (ESR). Since the ESR were so low for the medium/filtrate samples, suggesting extensive quenching, 100 μl of each medium/filtrate/scintillation fluid mixture was diluted into 3.5 ml additional scintillation fluid, and then recounted.

ELLA analyses were performed as in chapter 6. All results, both radioactive counts and mucus concentrations calculated from ELLA, were subjected to analysis of variance using a randomized block design. When significant main

effects were found, a Fisher's Least Significant Difference test was used to determine differences between groups.

Results

Dilution of radioactive samples did reveal some quenching of counts by the medium/filtrate solutions (Appendix D). However, following this dilution, the relationships between the groups remained the same, although variability within the groups was increased. The raw counts (corrected for background and ESR) in the medium/filtrate, and the percent of total counts in the medium/filtrate both demonstrated a significant increase in radiolabelled mucus secretion from explants incubated with cecal filtrates from rabbits with experimentally-induced ME as compared with those incubated with control filtrates. The explants incubated with either bethanechol in the control filtrate or with filtrates from rabbits that underwent cecal ligation without vessel ligation did not secrete a significantly greater amount of mucus into the medium.

As before, the ELLA demonstrated more mucus in the control filtrates initially, but not following explant incubation (figure 7.1). Following subtraction of the "blanks", these results were similar to those derived from ^{3}H-glucosamine incorporation (figure 7.2). Again, significantly more mucus was secreted from explants in the presence of cecal filtrates from rabbits with cecal ligation and mucus

hypersecretion than in the presence of filtrates from control rabbits ($P < 0.05$). In addition, according to the ELLA results, the increase seen with filtrates from cecal ligation rabbits (without mucus hypersecretion) is significantly greater than control ($P < 0.05$).

Discussion

It is surprising that no increase was seen in response to bethanechol, which has been shown to increase mucus secretion in rabbit jejunum (65) and rat colon (14). Other cholinergic agents, such as acetylcholine, also increase mucus secretion in the rabbit colon (217). However, in a pilot study (data not shown), explants were incubated with bethanechol in HBSS and still showed no effect; therefore this lack of response is probably not due to inhibitors in the cecal filtrate. Also, the consistency between ELLA and radioactive labelling results increases confidence in the ELLA model, and supports a lack of bethanechol effect.

Two different methods were used to quantitate mucus secretion in this study. The first involved incorporation of radioactive glucosamine into mucus *in vitro*, followed by stimulation of secretion into the medium/filtrate. The second involved an enzyme-linked assay, depending on the specificity of soybean agglutinin for N-acetylgalactosamine residues, which are a major component of mucus. Although both methods have several potential sources for error, there is good

agreement in the results obtained. The ELLA method showed significance for more subtle differences. Together, the results of these experiments support the hypothesis that there is a mucus secretagogue present in the cecal contents of rabbits with experimentally-induced mucoid enteropathy.

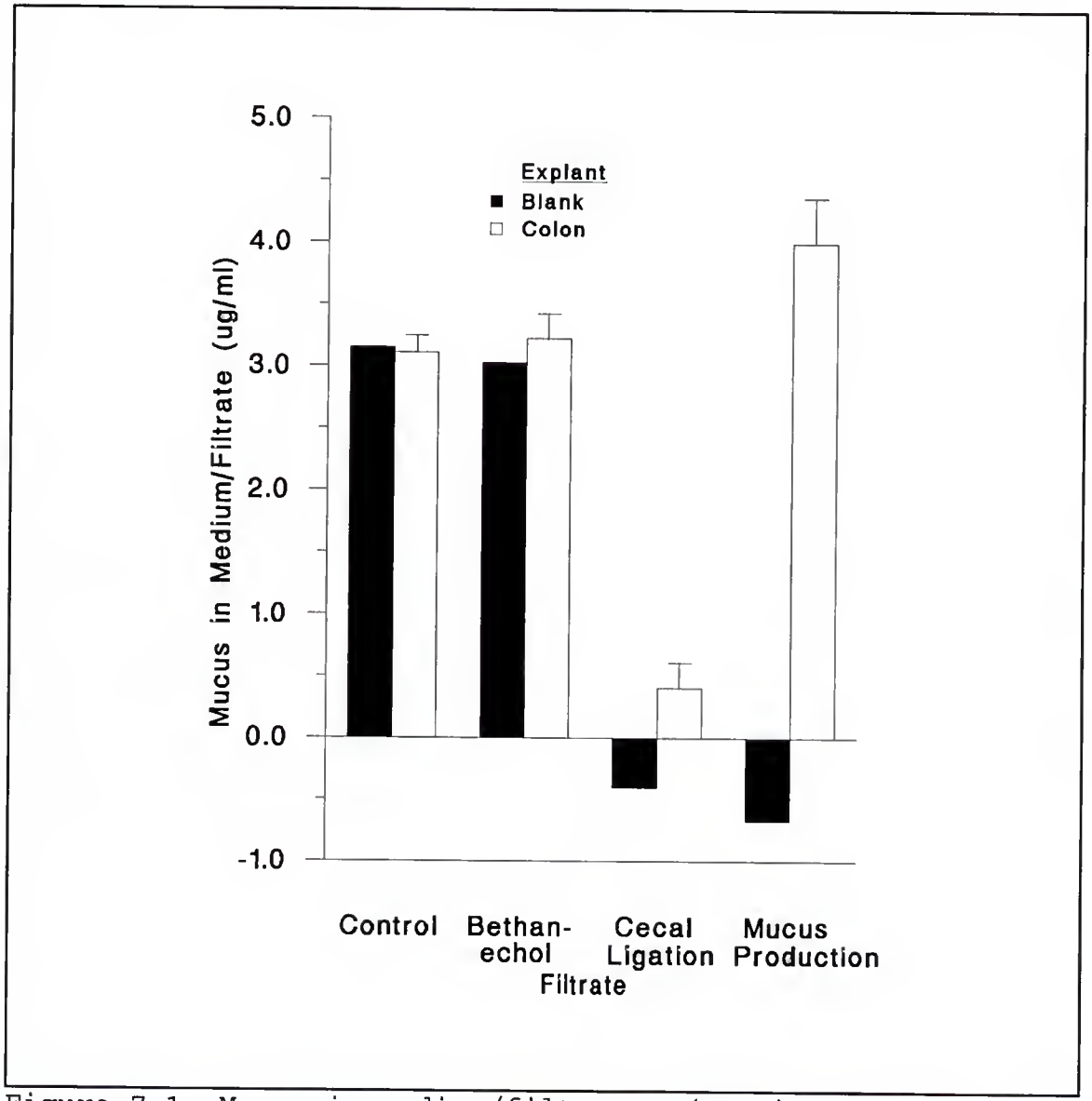

Figure 7.1. Mucus in medium/filtrates (ELLA).

Calculated mucus (porcine gastric mucin equivalents) in medium/filtrates, as measured by ELLA. Bars represent mean + SEM. N=1 (blanks) or 5 (with explants).

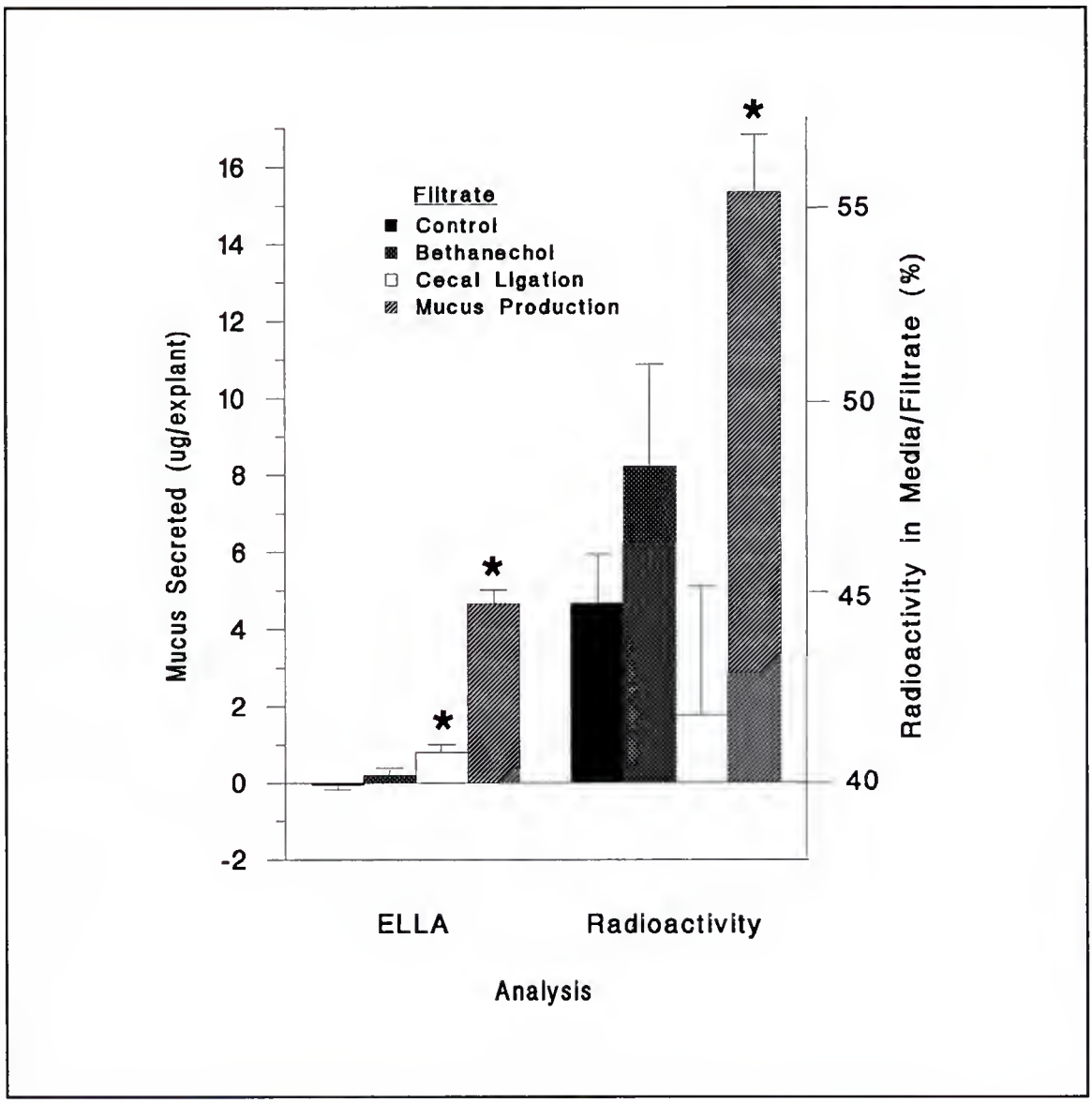

Figure 7.2. Comparison of ELLA and tracer secretion.

Mucus secretion determined by measuring the increase in mucus in the medium/filtrate following explant incubation (ELLA), or by the percentage of counts released into the medium/filtrate following incorporation of ^{3}H-glucosamine into mucus (Radioactivity). Bars represent mean + SEM. N = 5. * = significantly different from control ($P < 0.05$).

CHAPTER 8
PHYSICAL CHARACTERISTICS OF MUCUS SECRETAGOGUE

Introduction

The results obtained in Chapters 3, 6, and 7 provide strong evidence for the presence of mucus secretagogue activity in the cecal contents of rabbits with natural and experimental mucoid enteropathy. The next question is obvious: what is the physical nature of this secretagogue? This chapter describes the first step in defining the secretagogue: determination of protein or non-protein properties. Another question that is raised by the previous experiments is how a substance in the cecal contents can affect the colon when the cecum has been ligated. Therefore, since the most obvious route of transfer is through the blood, serum collected from cecal vein blood was tested for secretagogue activity.

Materials and Methods

Pooled cecal filtrate and serum samples were prepared. The "control" pool contained 6 ml from rabbit A1, and 19.5 ml from rabbit A12. The "ME" pool contained 8 ml from rabbit A5, and 17 ml from rabbit H7. In addition, cecal vein serum pools were prepared from 4.8 ml A1 and 5.5 ml A12 ("control"), and

5.5 ml A5 and 3.7 ml H7 ("ME"). Aliquots were taken from each pool: 4 ml for part A, which were used immediately, and 3 ml for part B, which were refrozen at -70°C. One aliquot for each experiment was untreated. Ammonium sulfate (2.24 g) was added to one 4 ml aliquot to give a final concentration of 85%; after 10 minutes the precipitate was collected by centrifugation and resuspended in 4 ml HBSS (39). Ten units of trypsin immobilized on beaded agarose (Sigma Chemical Co.) and 10 units of similar chymotrypsin beads were washed with 5 ml HBSS, divided in two equal portions, and each was resuspended in 0.2 ml HBSS, as described by the manufacturer. Aliquots of cecal filtrates were added to each of these enzyme preparations, and incubated at 25°C for 10 minutes. The beads were removed by centrifugation. Aliquots of both pooled filtrates and pooled serum were heated to 100°C for 30 minutes. The precipitated material was removed by centrifugation. Finally, filtrates were adjusted to pH 1.0 with HCl and kept at 25°C for 30 minutes, then returned to pH 7.0 with NaOH.

Three male SPF NZW rabbits, each weighing 2.3 kg were used as explant donors. In part A, two rabbits were sacrificed, and the single-banded section of each proximal colon was harvested. The intestinal segment was opened along the mesenteric border, washed in saline followed by HBSS/gentamicin, and 30 explants were collected from each rabbit using a 6 mm Baker's biopsy punch. Paired explants

were assigned to "control" and "ME" groups. The explants were incubated for 2 hours (pulse) at 37°C in 95% O_2/5% CO_2 in one ml Trowell's T8 medium containing 2 μCi ^{3}H-glucosamine and 100 μg gentamicin.

Each aliquot of treated or untreated filtrate, or serum, was added to an equal volume of Trowell's T8 medium containing 100 μg/ml gentamicin. One ml aliquots were placed in labelled polystyrene wells, including blank wells that would not contain explants. Following the pulse incubation, the explants from part A were washed by transferring them to individual wells containing 3 ml HBSS, and replacing the wash buffer 3 times. After washing, these explants were placed in the wells containing the appropriate medium/filtrate solution and incubated 1 hour at 37°C in 95% O_2/5% CO_2. Explants from the single rabbit in part B were collected from the colon in the same manner as for part A, and were immediately incubated with the corresponding medium/filtrate or medium/serum.

Then 20 μl of 5% N-acetylcysteine (final concentration 0.1%) was added to each well, and the medium/filtrate was collected. For part A, 100 μl of each medium/filtrate, as well as each explant, were transferred to a scintillation vial. Then 3.5 ml of scintillation fluid were added, and the samples were counted in a LKB 1218 Rackbeta. The remainder of each medium/filtrate from both parts was frozen in aliquots at -70°C.

Finally, 200 μl of each medium/filtrate from part A was precipitated by the addition of 0.112 g of ammonium sulfate (final concentration = 85%). Following centrifugation, the pellet was resuspended in 0.2 ml PBS and counted, to give a better estimate of radioactivity that had been incorporated into macromolecules. A direct ELLA was performed on each sample in both parts as described in Chapter 6. Selected samples were applied to 6% SDS-polyacrylamide gels, and either stained for protein with 0.25% Coomassie blue or analyzed for mucus using the Western blot technique described in Chapter 4.

All results, both radioactive counts and mucus concentrations calculated from ELLA, were subjected to analysis of variance using a randomized block design. When significant main effects were found, a Fisher's Least Significant Difference test was used to determine differences between groups. A Student's t test was used to compare serum results.

Results

The data obtained by measuring radioactivity in the medium/filtrate as a proportion of the total counts in both the medium/filtrate and the explant (figure 8.1, Appendix F), and that obtained by counting radioactivity that could be precipitated from the medium/filtrate by ammonium sulfate (figure 8.2) show similar overall trends. In the first case the only significant difference between control filtrates and

those from rabbits with experimental ME can be seen in untreated filtrates. On the other hand, for the precipitated samples, reflecting only incorporated ^{3}H-glucosamine and excluding any unincorporated radioactivity, the difference between control and experimental samples is significant for ammonium sulfate-insoluble filtrate fractions and cecal vein sera, but not the untreated filtrates. In neither case do the trypsin or chymotrypsin-treated filtrates appear to stimulate an increase in mucus secretion.

The results obtained by ELLA (figure 8.3, Appendix G) do not fully coincide with those obtained by measuring secretion of incorporated radioactivity. By this method, there is a significant increase in mucus secretion associated with filtrates from rabbits with experimental ME, whether the entire filtrates, the ammonium sulfate-insoluble fractions, or filtrates digested with trypsin or chymotrypsin are tested. However, secretagogue activity was abolished by heat or acid treatment (figure 8.4, Appendix H). When the data from part A are taken alone and analyzed by t-test, serum from rabbits with experimental ME appears to stimulate mucus secretion, but in much lesser amounts. In part B, no secretagogue activity was seen with either untreated or heat-treated serum.

Western blots (figure 8.5, lane 4) clearly demonstrated lectin-binding material in the stacking gel, verifying that the ELLA was measuring mucus. This material can be seen in medium/filtrate samples from ME rabbits following explant

incubation (lane 4), but not in the absence of an explant (lane 3). Material can be seen at approximately 144 kDa in control filtrates (lanes 1-2), but this is unchanged following explant incubation. There is minimal increase in staining of the mucus band in the stacking gel following incubation of control filtrates with colonic explants (lane 2).

Western blots further demonstrate that stimulation of mucus secretion from the explants occurs following incubation with trypsin and chymotrypsin-treated filtrates, as well as untreated filtrate and the ammonium sulfate-insoluble filtrate fraction (figure 8.6, lanes 6-9). No mucus could be seen in the ME serum sample following explant incubation (lane 10).

Discussion

The treatments performed on the cecal filtrate samples for this experiment were designed to determine whether or not the mucus secretagogue is a protein. Ammonium sulfate precipitates proteins, ribosomes, membrane fragments, and denatured proteins (39). The fact that the secretagogue is insoluble in ammonium sulfate suggests that it is a macromolecule. However, smaller molecules may be aggregated with larger molecules in the filtrate, so that they could also precipitate. We do have evidence of aggregation in the filtrates, in that the retentate following ultrafiltration with a Centricon®-100 showed the same banding pattern on an

SDS-polyacrylamide gel as the unfractionated filtrate (chapter 4).

The results following trypsin or chymotrypsin treatment were not consistent between the two assays. According to the ELLA, the secretagogue activity remained following protease treatment. This finding was supported by Western blot analysis of the medium/filtrate following explant incubation. Gels stained for protein with Coomassie blue revealed the same banding pattern with or without protease treatment, suggesting that in fact the proteases were not active in the medium/filtrates. Therefore, we would predict activity in the trypsin and chymotrypsin samples, whatever the nature of the secretagogue. It is possible that the radioactivity incorporation method was not sensitive enough to demonstrate mucus secretion in these samples. This lack of sensitivity has been reported previously (49).

Protease treatments were employed to determine whether or not the secretagogue is a protein. Unfortunately, trypsin and chymotrypsin appeared to have no effect on any of the proteins in the cecal filtrates. In addition, incubation of filtrates with 2 units/ml pronase for 30 minutes at 30°C had no effect (figure 8.7), suggesting the presence of protease inhibitors in the cecal filtrates. Consequently, it was not possible in this study to state definitively that the secretagogue is a protein. However, the secretagogue is both heat- and acid-

labile, giving further evidence that it is a large molecule, and the most likely candidate is still a protein.

Cecal vein serum was also tested in the explant system, to determine if the secretagogue is transmitted by serum, as previously postulated (236). These results are ambiguous. An increase in macromolecular radioactivity was seen in medium/serum following explant incubation with serum from rabbits with experimental ME. Using a t-test to evaluate the ELLA results separately from the filtrate-associated samples also demonstrated an increase. However, the increase was a degree of magnitude lower than that seen with the filtrate, and the mucus concentration was below the limit of detection of the Western blot. Following refreezing of the serum, even that minor activity was eliminated. It is possible that a very small amount of the secretagogue is present in the serum, and that by chance the radioactivity results were able to detect it. Alternatively, the radioactivity increase may be associated with a glycoprotein other than mucus.

There is another explanation for these discrepancies that may be considered. The radioactivity incorporation method only measures release of newly synthesized mucin. On the other hand, the ELLA measures release of all mucin--that which is newly synthesized, and that which was formed prior to the death of the animal and has been stored. It is possible that two mechanisms of increased secretion are involved. One is not protease-sensitive under these conditions, is not present

in the serum, and results in the accelerated release by "compound exocytosis" of stored mucin in the manner of acetylcholine and mustard oil (217). The other which is trypsin- and chymotrypsin-sensitive, and is in the serum would either accelerate the rate of "baseline" secretion (167, 184), or would increase the rate of ^{3}H-glucosamine incorporation into mucin, such that a greater amount of the mucin that is secreted in a baseline manner contains the radioactive label. The increase in incorporation at first appears less likely, since the explants were washed prior to incubation with the medium/filtrates; but free glucosamine could still be present and available for incorporation within the cells, or associated with the explants.

From these experiments it is apparent that a large macromolecule, most likely a protein, could be responsible for the majority of the stimulated mucus secretion. The exact nature of the secretagogue is still not certain, however, or even if the secretagogue is a single entity. Alternative techniques will have to be developed to distinguish mucus synthesis from baseline secretion from compound exocytosis.

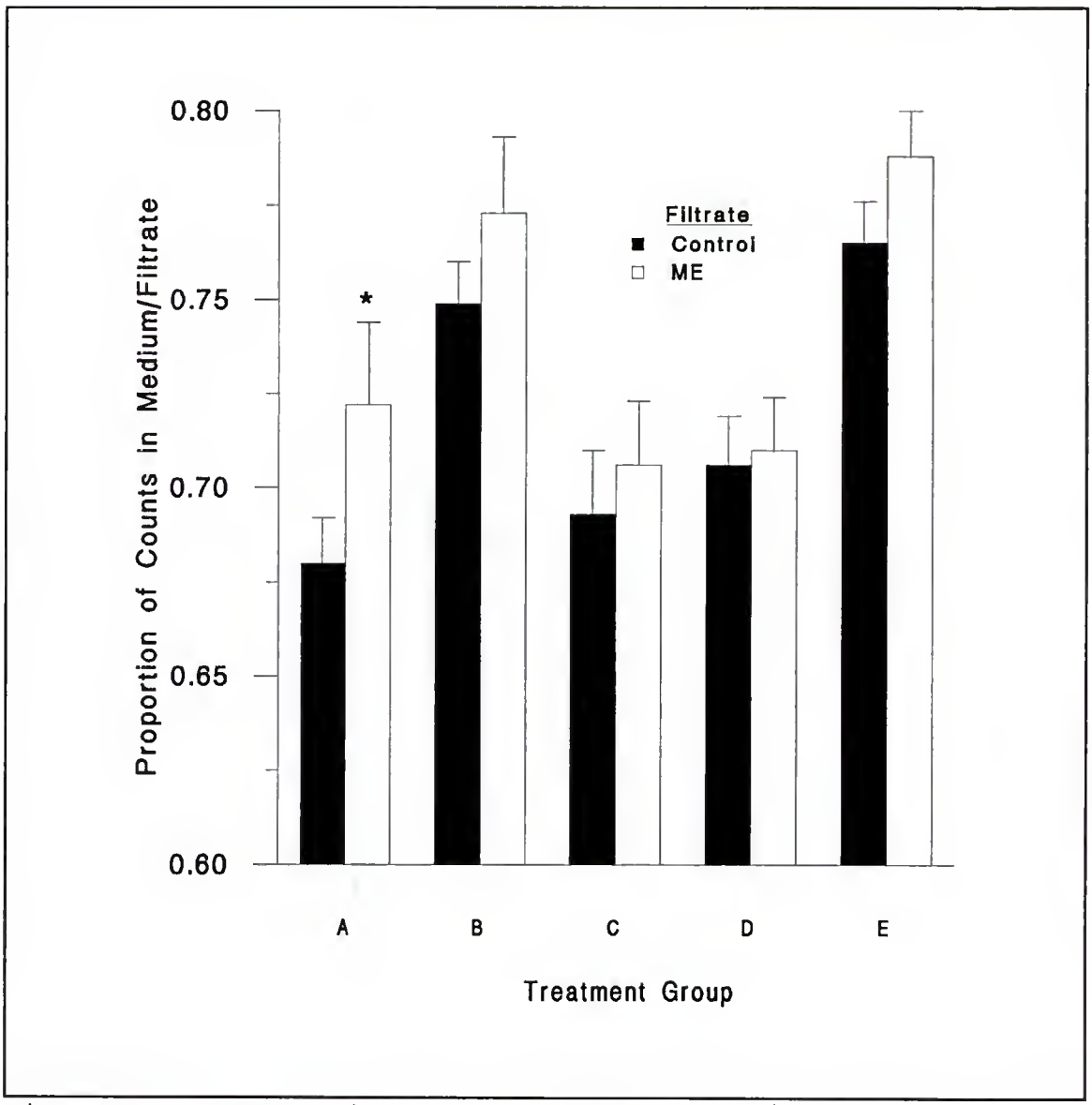

Figure 8.1. Proportion of tracer in medium/filtrate.

Following incubation with ^{3}H-glucosamine-labelled explants, counts in 100 μl of medium/filtrate were multiplied by 10 and divided by this number plus the counts in the explant (total counts). "Control" indicates filtrates collected from healthy rabbits; "ME" indicates filtrates collected from rabbits with experimental mucoid enteropathy. Treatment groups are A) untreated filtrate, B) fraction of filtrate insoluble in 85% ammonium sulfate, C) trypsin-treated filtrate, D) chymotrypsin-treated filtrate, and E) cecal vein serum. Bars represent mean + SEM. N = 6. * = significantly different from control ($P < 0.05$).

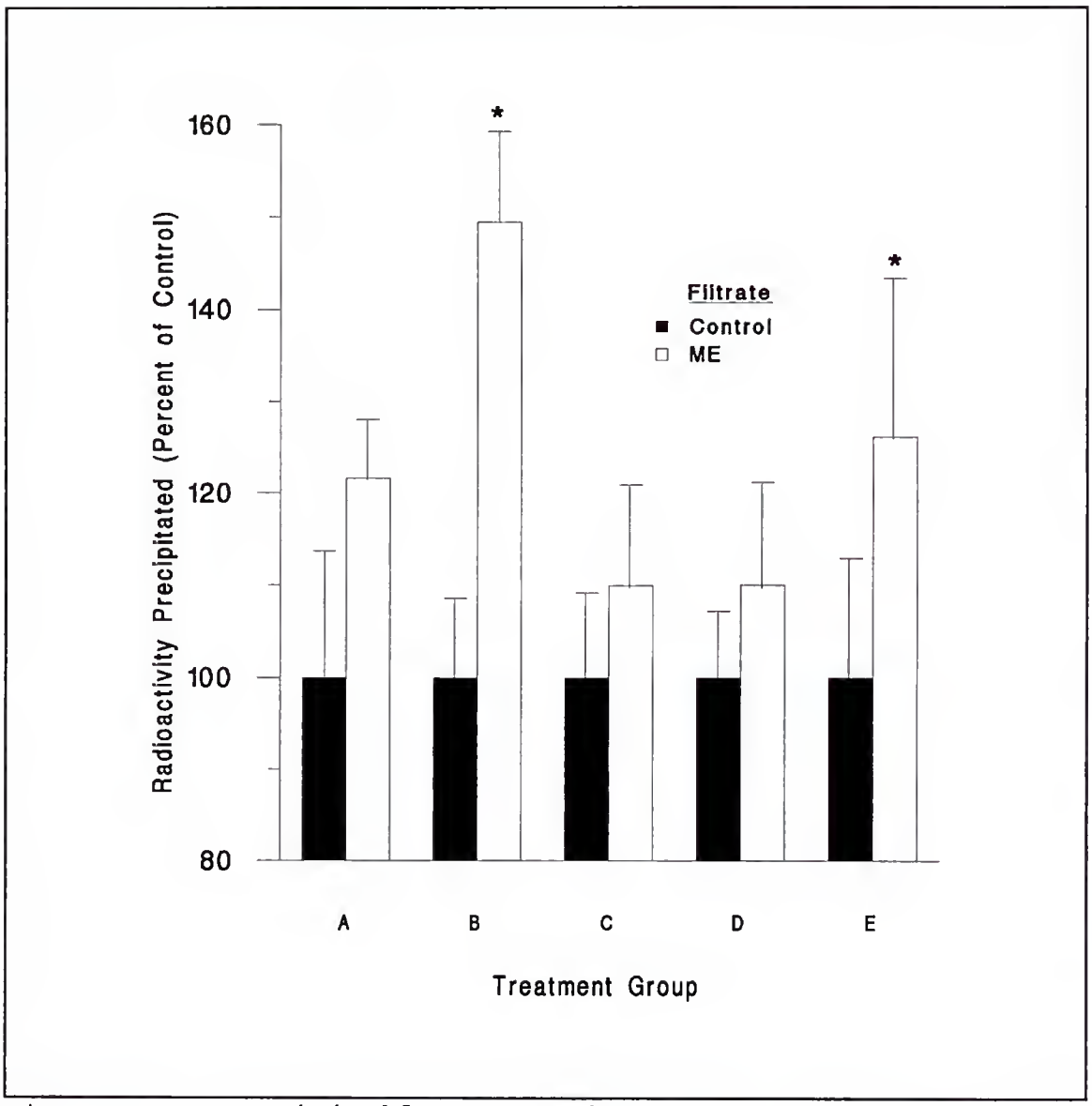

Figure 8.2. Precipitable secreted tracer.

Macromolecules (ammonium sulfate insoluble material) in medium/filtrates following incubation with ^{3}H-glucosamine-labelled explants. Results are shown as percent of control values. "Control" indicates filtrates collected from healthy rabbits; "ME" indicates filtrates collected from rabbits with experimental mucoid enteropathy. Treatment groups are A) untreated filtrate, B) fraction of filtrate insoluble in 85% ammonium sulfate, C) trypsin-treated filtrate, D) chymotrypsin-treated filtrate, and E) cecal vein serum. Bars represent mean + SEM. N = 6. * = significantly different from control ($P < 0.05$).

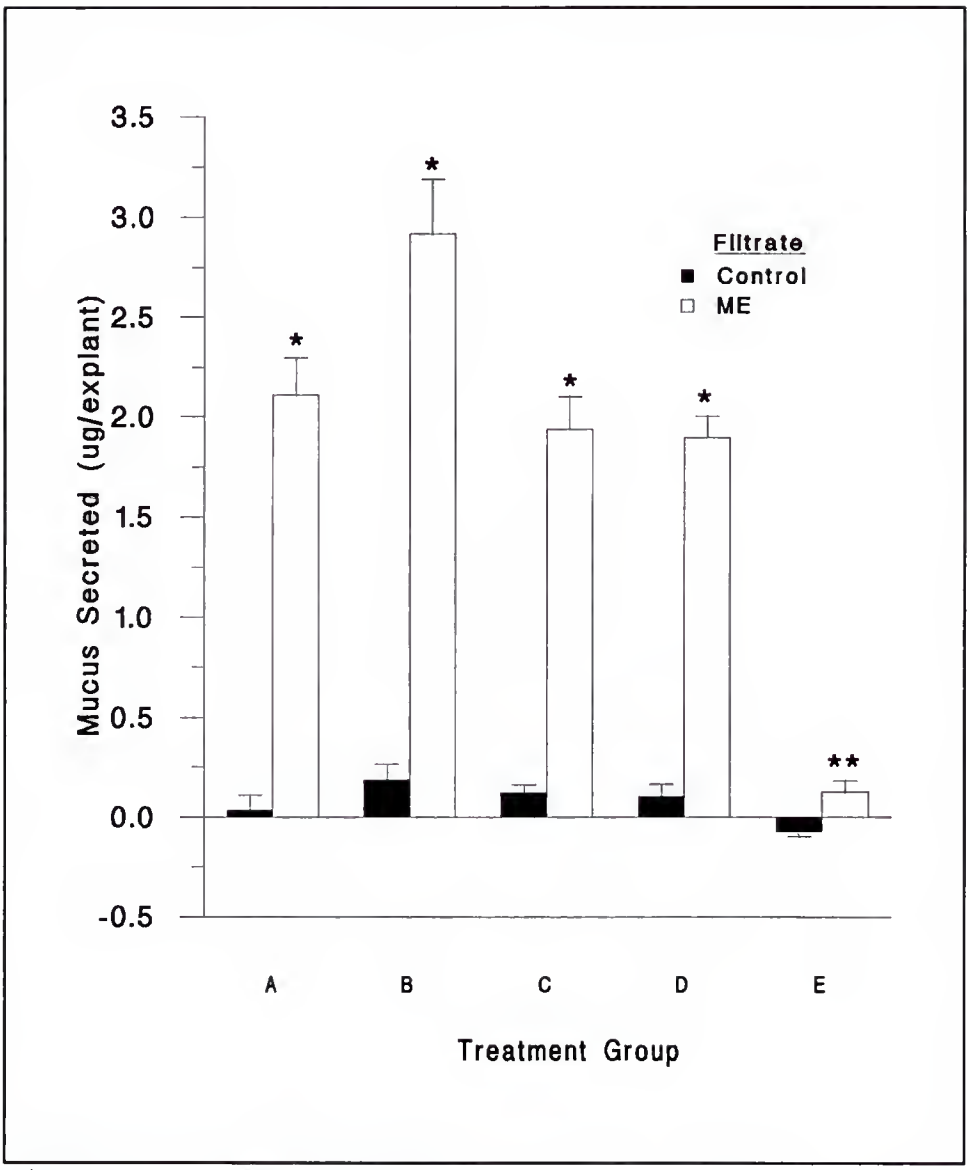

Figure 8.3. Mucus secretion, part A (ELLA). Mucus secretion determined by subtracting the amount of mucus in the medium/filtrate incubated without explants from the amount incubated with explants, as measured by ELLA. "Control" indicates filtrates collected from healthy rabbits; "ME" indicates filtrates collected from rabbits with experimental mucoid enteropathy. Treatment groups are A) untreated filtrate, B) fraction of filtrate insoluble in 85% ammonium sulfate, C) trypsin-treated filtrate, D) chymotrypsin-treated filtrate, and E) cecal vein serum. Bars represent mean + SEM. N = 6. * = significantly different from control ($P < 0.05$) by ANOVA; ** = significantly different from control by t-test ($P < 0.05$).

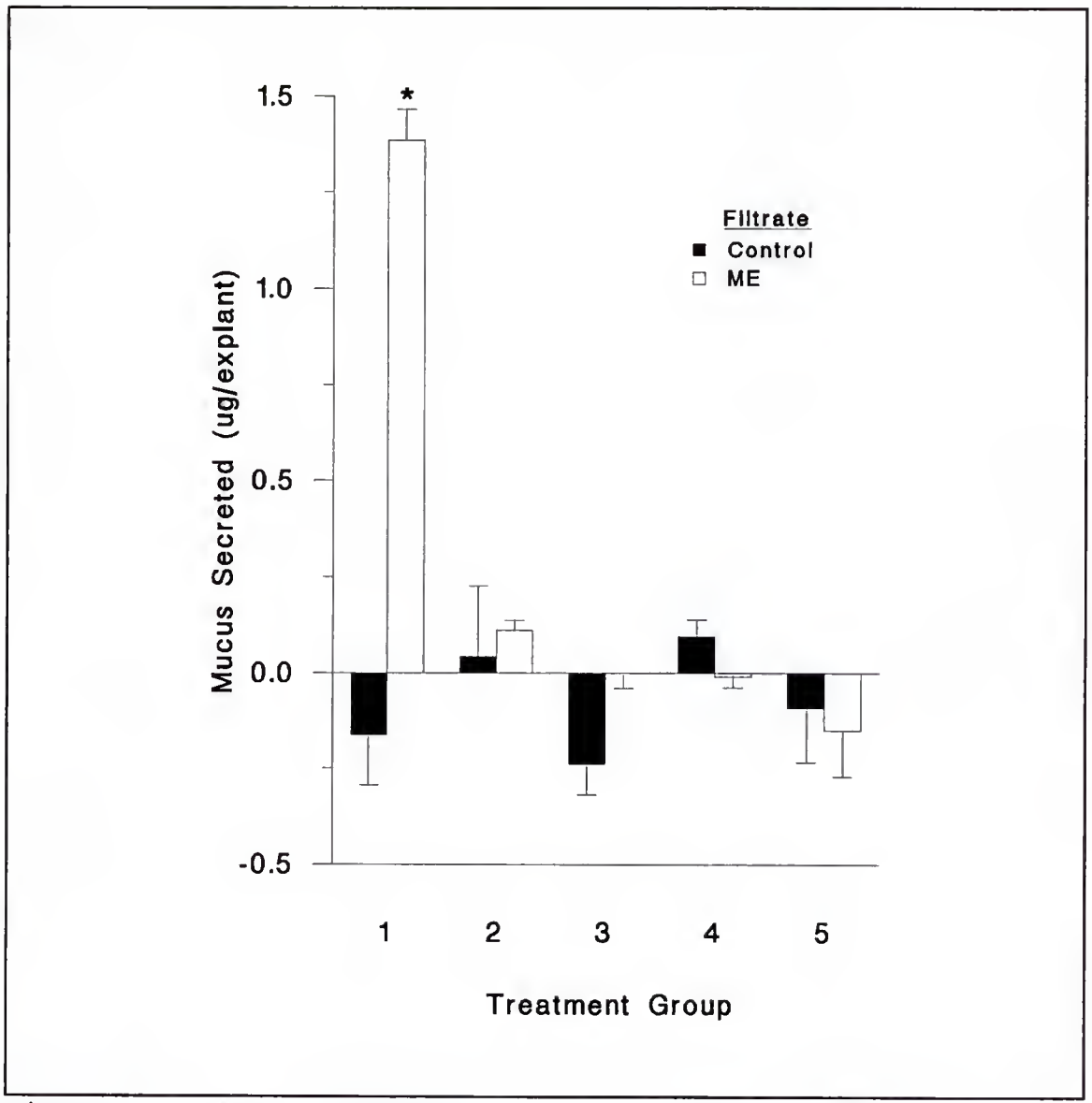

Figure 8.4. Mucus secretion, part B (ELLA).

Mucus secretion determined by subtracting the amount of mucus in the medium/filtrate incubated without explants from the amount incubated with explants, as measured by ELLA. "Control" indicates filtrates or sera collected from healthy rabbits; "ME" indicates filtrates or sera collected from rabbits with experimental mucoid enteropathy. Treatment groups are 1) untreated filtrate, 2) filtrate heated to 100°C for 30 minutes, 3) filtrate acidified to pH 1 for 30 minutes, 4) untreated cecal vein serum, and 5) cecal vein serum heated to 100°C for 30 minutes. Bars represent mean + SEM. N = 5. * = significantly different from control ($P < 0.05$).

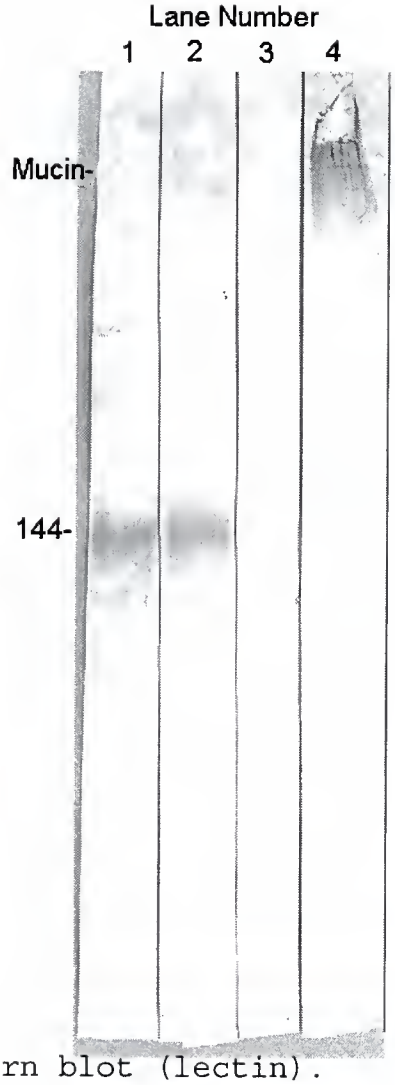

Figure 8.5. Western blot (lectin).

Lanes containing: 1) pooled control medium/filtrate, 2) same filtrate following incubation with colonic explant, 3) pooled experimental ME medium/filtrate, and 4) same filtrate following incubation with colonic explant labelled with soybean agglutinin.

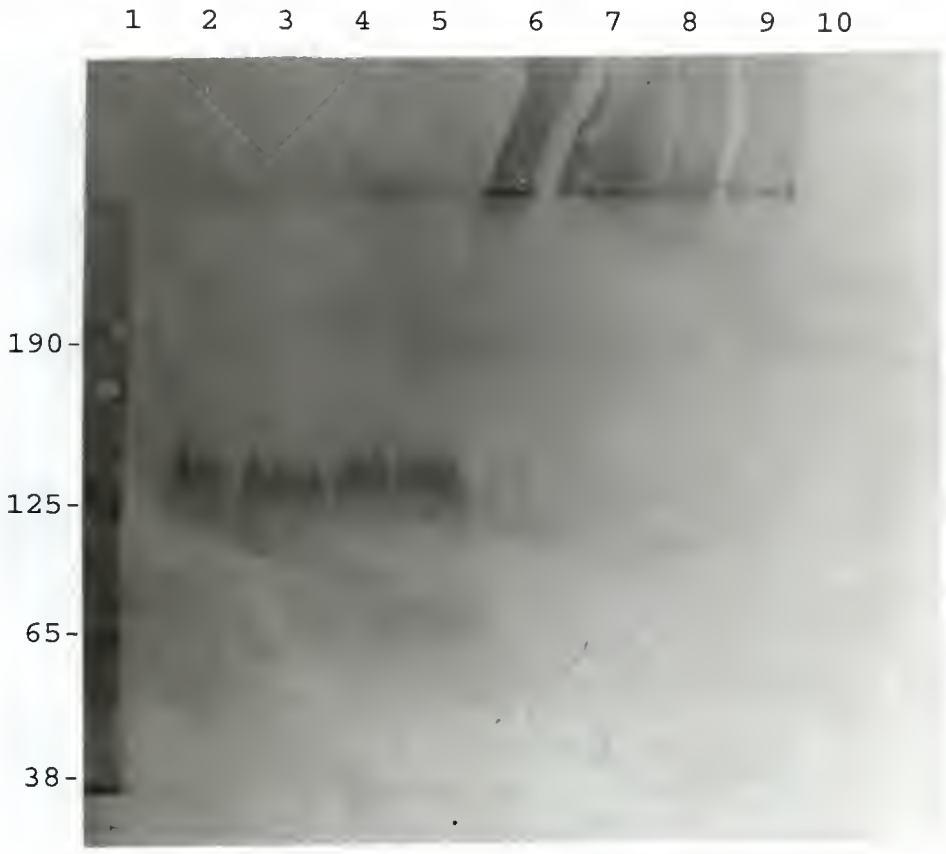

Figure 8.6. Western blot (lectin).

Lane 1 contains molecular weight standards. Lanes contain control (2-5) or ME (6-9) medium/filtrates that are untreated (2,6), insoluble in ammonium sulfate (3,7), trypsin-treated (4,8), or chymotrypsin-treated (5,9); or ME cecal vein serum (10), all following incubation with colonic explants, and labelled with soybean agglutinin.

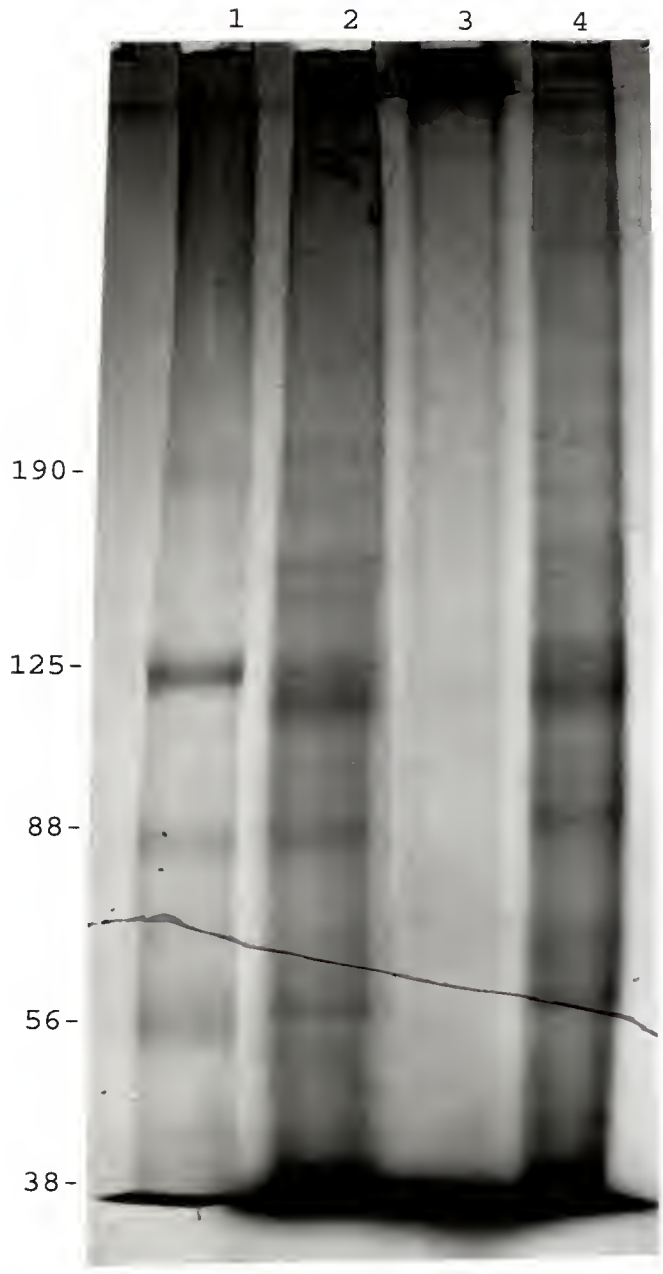

Figure 8.7. SDS-polyacrylamide gel.

Lanes containing: 1) molecular weight standards, 2) experimental ME cecal filtrate, 3) same filtrate heated to 100°C for 30 minutes, and 4) same filtrate treated with 2 Units/ml pronase for 30 minutes stained for protein with Coomassie blue.

CHAPTER 9
CONCLUSIONS

The goal of this project was to study the etiology of a serious, not uncommon disease of rabbits. Mucoid enteropathy is unique to the rabbit, and so has not been the subject of intense interest. Therefore, the first task was to establish a reproducible model of the disease to provide samples for further study. The cecal ligation model developed by Toofanian and Targowski (229, 235) was reported to induce a mucoid enteropathy-like syndrome in 70% of rabbits. In this present study the success rate was only 12.5% in SPF rabbits. However, incorporation of the cecal vessels into the ligature increased the incidence of gross mucus hypersecretion to 100% in SPF rabbits. Yet, this also caused severe necrosis and inflammation of the cecum, which is not seen in natural cases of mucoid enteropathy.

The histologic changes seen following cecal ligation were inconsistent, but they paralleled the inconsistent changes reported for the natural disease. Inflammation of the distal colon is seen in both natural and experimental disease, as is mild necrosis of the cecum (142, 238). Therefore, cecal ligation appears to represent a valid model of mucoid enteropathy.

The cecal filtrates from rabbits with both natural and experimental mucoid enteropathy induced mucus secretion from colonic explants from healthy rabbits. Secretion from ileal explants was also demonstrated following incubation with a filtrate from a rabbit with the natural disease. However, the variability between ileal explants obscured any potential stimulation when the filtrates from rabbits with the experimental disease were tested. Thus, ileal explants were not used to assess secretagogue activity for these studies.

There was significant variation between the filtrates in secretagogue activity. In particular, the filtrate from the rabbit (A5) that developed gross mucus hypersecretion without vessel ligation demonstrated the strongest activity. Two explanations for this seem plausible: 1) if the secretagogue is of host origin, this rabbit may have had a genetic predilection to produce the secretagogue and develop the disease; 2) if the secretagogue is of microbial origin, this rabbit may have harbored more of the organism prior to surgery, allowing for quicker overgrowth following even the less radical surgery.

The secretagogue appears to be a macromolecule, most likely a protein. Of the known intestinal mucus secretagogues (chapter 2), the chemical properties are most consistent with microbial enterotoxins, such as cholera and *E. coli* labile toxin, or products of inflammation, such as interleukin-1, immune complexes or MMS-68. Based on a comparison of

histology and secretagogue activity, it seems unlikely that the secretagogue is an inflammatory mediator. There was no apparent relationship between the degree of inflammation seen following surgery, and the stimulation of mucus secretion from explants. Furthermore, inflammation is not always present in the natural disease.

On the other hand, cecectomy does not induce mucoid enteropathy and instillation of tetracycline into the ligated cecum prevents development of the disease, suggesting a bacterial etiology (235). Cecal ligation in conjunction with inoculation of *Vibrio cholerae* organisms results in mucoid diarrhea in the RITARD model (222). This demonstrates that rabbits are susceptible to cholera toxin if the concentration is high enough. Also, cholera toxin and *E. coli* LT are stimulated, not destroyed, by trypsin. Therefore, resistance of enterotoxins to digestive enzymes is not unexpected.

It appears that either very small amounts of the secretagogue are carried in the serum, or that there is a second factor in the serum that increases production or baseline secretion of mucus. Thus, the serum is not a useful fluid for study of the secretagogue, but it is possible that the secretagogue present in the ligated cecum is transported via the bloodstream to the colon to stimulate goblet cells (236).

This study has added to our understanding of mucoid enteropathy of rabbits. The existence of a secretagogue has

been demonstrated, which can be the focus of further study. This secretagogue is present in rabbits with the natural disease, and can be induced experimentally. Physical separation techniques can be used to identify it. Once it is isolated, antibodies can be constructed and used to localize its source. Once that is known, rational therapy may be possible.

Future Directions

This study has only looked at mucus secretion, which may be the last step in the pathogenesis of this disease. If this is a microbial toxin, what is the microbe? How do the rabbits become infected? What makes some animals resistant? Is the microbe directly pathogenic, or does there have to be an additional insult or disruption of microbial flora? If the secretagogue is of host origin, from whence does it come? What regulates it? What is the secretagogue of the secretagogue? Although progress has been made, there is still a great deal of work to be done to truly understand this disease.

APPENDIX A
DATA TABLES FOR CHAPTER 3

Mucus in media/filtrates, measured by ELLA

(μg/ml porcine gastric mucin equivalents)

No explant:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
	-0.333	0.410	0.057
	0.033		-0.202
	0.031		-0.185
	0.339		-0.032
	0.144		0.041
Mean	0.043	0.410	-0.064
n	5	1	5
SEM	0.098	N/A	0.049

Mucus in media/filtrates, measured by ELLA

(μg/ml porcine gastric mucin equivalents)

Ileum explants:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
P8	0.733		2.148
P9	0.499		0.558
	1.049		1.252
	1.005		1.283
			1.080
			1.049
			1.293
			1.336
R1	0.429	0.749	0.566
		0.666	0.502
R2	0.454	0.744	1.078
		0.512	0.881
R3	0.376	0.571	0.646
		0.476	0.585
Mean	0.649	0.620	1.014
n	7	6	14
SEM	0.245	0.044	0.115

Mucus in media/filtrates, measured by ELLA

(μg/ml porcine gastric mucin equivalents)

Colon explants:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
P8	0.078		0.084
P9	0.417		0.883
	0.223		0.882
	0.594		0.438
R1	0.505	0.454	0.317
		0.581	0.366
R2	0.232	0.634	0.341
		0.534	0.334
R3	0.302	0.683	0.566
		0.410	0.454
Mean	0.336	0.549	0.467
n	7	6	10
SEM	0.063	0.039	0.075

Mucus secreted into media/filtrate (μg/ml)

Ileum explants:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
P8	1.066		2.091
P9	0.466		0.760
	0.710		1.283
	0.666		1.314
			1.112
			1.081
			1.325
			1.304
R1	0.285	0.339	0.524
		0.256	0.461
R2	0.310	0.334	1.037
		0.102	0.839
R3	0.232	0.161	0.605
		0.066	0.544

Mucus secreted into media/filtrate (μg/ml)

Colon explants:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
P8	0.411		0.026
P9	0.384		1.085
	0.192		1.068
	0.563		0.624
R1	0.361	0.044	0.276
		0.171	0.324
R2	0.088	0.224	0.300
		0.124	0.293
R3	0.159	0.273	0.524
		0.000	0.412

Explant weights (mg)

Filtrate

	Control P3	Control P8	Mucoid enteropathy P4
Ileum explants:			
P8	31.00		39.00
P9	29.24		35.10
R1	21.67	22.37	19.83
		18.55	28.46
R2	24.87	24.56	25.83
		22.20	24.69
R3	19.76	13.59	18.30
		21.79	18.00
Colon explants:			
P8	34.57		41.93
P9	35.54		42.87
R1	19.40	21.64	22.51
		20.95	25.08
R2	25.16	18.78	21.27
		19.40	19.59
R3	24.19	25.55	22.24
		25.06	23.33

Mucus secreted (ng/mg explant tissue)

Ileum explants:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
P8	34.39		53.61
P9	15.93		21.65
R1	13.17	15.16	26.45
		13.81	16.20
R2	12.46	13.61	40.14
		4.61	33.99
R3	11.73	11.85	33.06
		3.02	30.22
Mean	17.53	10.34	31.91
n	5	6	8
SEM	4.27	2.12	4.07

	Control	Mucoid Enteropathy
Mean	13.61	31.91*
n	11	8
SEM	2.41	4.07

* t = 3.86, P = 0.002, significantly different from control

Mucus secreted (ng/mg explant tissue)

Colon explants:

	Filtrate		
	Control P3	Control P8	Mucoid enteropathy P4
P8	11.89		0.63
P9	10.81		25.31
R1	18.61	2.03	12.25
		8.15	12.94
R2	3.49	11.95	14.11
		6.41	14.94
R3	6.55	10.69	23.58
		0.00	17.67
Mean	10.27	6.54	15.18
n	5	6	8
SEM	2.17	1.93	2.69

	Control	Mucoid Enteropathy
Mean	8.24	15.18*
n	11	8
SEM	1.60	2.69

* t = 2.21, P = 0.05, significantly different from control

APPENDIX B
DATA TABLES FOR CHAPTER 5

Animal Number	Survival (days)	Initial weight (kg)	Final weight (kg)	Weight gain (kg)	Weight gain/day (kg)	Cecal contents (g)	Cecal contents (ml)	Filtrate retained (%)	Dry weight (mg/ml)	Full cecum/body wt (g/kg)	Empty cecum/body wt (g/kg)
Control:											
A01		2.0	2.015	0.0	0.005	64	50	35.0%	36.67	57.07	25.31
A02		1.9	1.978	0.1	0.019	103	100	26.7%	72.22	70.78	18.71
A04		2.1	2.368	0.3	0.027	49	30	37.5%	44.44	46.45	25.76
A11		1.9	2.205	0.3	0.038	87	80	26.9%	48.00	73.47	34.01
A12		2.0	2.331	0.3	0.033	67	60	31.8%	36.67	63.92	35.18
Ligation/no vessel:											
A03	10	2.0	1.857	-0.1	-0.014	18	20	50.0%	44.00	27.46	17.77
A05	4	1.9	1.563	-0.3	-0.084	37	40	27.8%	33.00	53.74	30.07
A06	4	1.7	1.512	-0.2	-0.047	28	30	45.0%	28.00	41.01	22.49
A10	10	2.2	1.890	-0.3	-0.031	43	40	27.8%	48.00	38.62	15.87
H1	4	1.40	1.092	-0.3	-0.077	23	20	42.9%	37.00	43.96	22.89
H2	4	1.32	1.289	-0.0	-0.008	42	40	22.2%	53.00	45.77	13.19
H3	4	1.50	1.370	-0.1	-0.032	41	40	22.2%	39.00	48.18	18.25
H4	4	1.56	1.456	-0.1	-0.026	36	30	43.8%	39.00	41.21	16.48

Animal Number	Survival (days)	Initial weight (kg)	Final weight (kg)	Weight gain (kg)	Weight gain/day (kg)	Cecal contents (g)	Cecal contents (ml)	Filtrate retained (%)	Dry weight (mg/ml)	Full cecum/body wt (g/kg)	Empty cecum/body wt (g/kg)
Ligation/vessel:											
A07	3	1.7	1.692	-0.0	-0.049	58	50	25.0%	36.00	63.83	29.55
A08	3	2.0	1.615	-0.4	-0.128	51	50	35.0%	64.00	56.97	25.39
A09	5	1.7	1.475	-0.2	-0.178	15	15	61.5%	12.00	48.14	37.97
H05	3	2.30	1.905	-0.4	-0.132	32	30	40.0%	33.68	43.57	26.77
H06	3	2.08	1.786	-0.3	-0.098	33	35	41.2%	47.00	35.27	16.80
H07	3	2.12	1.821	-0.3	-0.100	31	30	43.8%	39.00	35.69	18.67

All animals		Initial weight (kg)	Final weight (kg)	Weight gain (kg)	Weight gain/day (kg)	Cecal contents (g)	Cecal contents (ml)	Filtrate retained (%)	Dry weight (mg/ml)	Full cecum/body wt (g/kg)	Empty cecum/body wt (g/kg)
Control	mean	1.98	2.18	0.20	0.025	74.00	64.00	31.6%	47.60	62.34	27.79
	std	0.08	0.18	0.14	0.013	21.12	27.02	4.8%	14.62	10.93	6.82
Ligation	mean	1.70	1.50	-0.19	-0.040	33.50	32.50	35.2%	40.13	42.49	19.63
(no vessel)	std	0.31	0.27	0.11	0.028	9.40	8.86	11.3%	8.04	7.71	5.33
Ligation	mean	1.98	1.72	-0.27	-0.114	36.67	35.00	41.1%	38.61	47.24	25.86
(vessel)	std	0.24	0.16	0.14	0.043	15.47	13.42	12.0%	17.05	11.49	7.68
Ligation	mean	1.82	1.59	-0.23	-0.072	34.86	33.57	37.7%	39.48	44.53	22.30
(both)	std	0.31	0.25	0.13	0.051	11.93	10.64	11.6%	12.14	9.42	9.89

Study animals		Initial weight (kg)	Final weight (kg)	Weight gain (kg)	Weight gain/day (kg)	Cecal contents (g)	Cecal contents (ml)	Filtrate retained (%)	Dry weight (mg/ml)	Full cecum/body wt (g/kg)	Empty cecum/body wt (g/kg)
Control	mean	1.98	2.18	0.20	0.025	74.00	64.00	31.58%	47.60	62.34	27.79
	std	0.08	0.18	0.14	0.013	21.12	27.02	4.81%	14.62	10.93	6.82
Ligation	mean	1.82	1.62*	-0.20*	-0.037**	33.60*	34.00*	34.56%	41.20	41.32**	19.88
(no vessel)	std	0.33	0.25	0.13	0.031	10.55	8.94	12.16%	10.43	12.99	7.53
Ligation	mean	2.04	1.76*	-0.28*	-0.101*	41.00*	39.00*	36.99%	43.94	47.07	23.44
(vessel)	std	0.22	0.11	0.16	0.033	12.59	10.25	7.42%	12.29	12.84	5.46

* = significantly different from control ($P < 0.05$); ** = ($P < 0.05$)

ANALYSIS OF VARIANCE TABLES FOR CHAPTER 5

Initial weight (kg)

Source	SS	df	MS	F
Between	0.124	2	0.062	1.12
Within	0.668	12	0.056	
Total	0.793	14		

Necropsy weight (kg)

Source	SS	df	MS	F	LSD
Between	0.839	2	0.419	11.67*	0.26
Within	0.431	12	0.036		
Total	1.270	14			

Weight gain (kg)

Source	SS	df	MS	F	LSD
Between	0.654	2	0.327	16.13**	0.20
Within	0.243	12	0.020		
Total	0.898	14			

Weight gain/day (kg)

Source	SS	df	MS	F	LSD
Between	0.0396	2	0.019788	26.90**	0.037
Within	0.0088	12	0.000736		
Total	0.0484	14			

* $P < 0.005$; **$P < 0.001$

Weight cecal contents collected (g)

Source	SS	df	MS	F	LSD
Between	4627	2	2313	9.70*	21.3
Within	2863	12	239		
Total	7490	14			

Volume of cecal contents collected (ml)

Source	SS	df	MS	F	LSD
Between	2583	2	1292	4.23***	24.1
Within	3660	12	305		
Total	6243	14			

Filtrate retained following processing of cecal contents (%)

Source	SS	df	MS	F
Between	0.00732	2	0.003662	0.49
Within	0.09042	12	0.007535	
Total	0.09775	14		

Dry weight (mg/ml)

Source	SS	df	MS	F
Between	103	2	52	0.33
Within	1895	12	158	
Total	1998	14		

* P < 0.005; ***P < 0.05

Full cecum/body weight ratio

Source	SS	df	MS	F	LSD
Between	1180	2	590	4.69***	15.5
Within	1511	12	126		
Total	2691	14			

Empty cecum/body weight ratio

Source	SS	df	MS	F
Between	157	2	79	1.96
Within	481	12	40	
Total	638	14		

***P < 0.05

APPENDIX C
DATA TABLES FOR CHAPTER 6

Mucus in undiluted media/filtrate (μg/ml)

Control filtrates: Ileum explants

	A1	A2[a]	A4	A11	A12	Mean	SEM
No explant	2.908	30.199	0.842	0.440	1.052	7.088	5.793
H8	3.837	29.578	3.837	2.975	2.190	8.484	5.283
H9	1.904	31.351	1.231	0.760	1.074	7.264	6.025
H10	2.131	27.470	3.893	1.779	2.143	7.485	5.010
H11	3.943	30.514	4.447	5.157	3.734	9.559	5.245
H12	2.647	30.139	1.610	2.783	1.858	7.807	5.587
Mean (explants)	2.892	29.811	3.004	2.691	2.200	8.119	2.228
SEM	0.425	0.652	0.658	0.733	0.432		

Control filtrates: Colon explants

	A1	A2[a]	A4	A11	A12	Mean	SEM
No explant	2.908	30.199	0.842	0.440	1.052	7.088	5.793
H8	2.763	30.369	1.134	1.134	1.202	7.320	5.771
H9	2.545	29.612	0.952	0.789	1.048	6.989	5.664
H10	2.361	27.430	1.089	1.053	1.120	6.611	5.211
H11	2.159	26.673	1.122	1.015	1.217	6.437	5.063
H12	1.920	31.978	1.212	0.843	1.376	7.466	6.130
Mean (explants)	2.350	29.212	1.102	0.967	1.193	6.965	2.280
SEM	0.147	0.969	0.043	0.065	0.055		

Mucus in undiluted media/filtrate (μg/ml)

Ligation/no vessel filtrates: Ileum explants

	A3	A5	A6	A10	H2	Mean	SEM
No explant	0.519	0.236	0.823	0.002	0.031	0.322	0.156
H8	2.129	2.344	1.994	0.545	1.620	1.726	0.318
H9	0.760	3.229	1.366	0.029	0.719	1.211	0.545
H10	0.955	2.344	1.095	0.265	1.150	1.162	0.335
H11	1.487	3.038	2.378	1.627	3.976	2.501	0.462
H12	0.552	2.534	1.535	0.194	1.563	1.276	0.414
Mean (explants)	1.177	2.698	1.673	0.532	1.806	1.577	0.201
SEM	0.284	0.184	0.229	0.286	0.566		

Ligation/no vessel filtrates: Colon explants

	A3	A5	A6	A10	H2	Mean	SEM
No explant	0.519	0.236	0.823	0.002	0.031	0.322	0.156
H8	0.712	3.437	1.255	0.054	1.015	1.295	0.572
H9	0.530	5.616	1.417	0.103	1.013	1.736	0.995
H10	0.479	4.205	1.748	0.143	1.026	1.520	0.724
H11	0.690	3.696	1.514	0.360	1.511	1.554	0.582
H12	0.489	3.208	1.822	0.200	0.943	1.332	0.543
Mean (explants)	0.580	4.032	1.551	0.172	1.102	1.487	0.289
SEM	0.050	0.429	0.105	0.053	0.103		

Mucus in undiluted media/filtrate (μg/ml)

Ligation/vessel filtrates: Ileum explants

	A7	A8	H5	H6	H7	Mean	SEM
No explant	1.897	0.137	0.152	0.031	0.122	0.468	0.358
H8	4.890	1.125	2.131	0.845	1.706	2.139	0.723
H9	1.689	0.174	0.279	0.294	0.654	0.618	0.280
H10	2.478	0.450	0.620	0.271	0.570	0.878	0.405
H11	5.629	1.624	2.629	1.689	2.329	2.780	0.737
H12	2.748	0.195	1.119	0.520	1.274	1.171	0.440
Mean (explants)	3.487	0.714	1.355	0.724	1.307	1.517	0.279
SEM	0.753	0.285	0.446	0.262	0.330		

Ligation/vessel filtrates: Colon explants

	A7	A8	H5	H6	H7	Mean	SEM
No explant	1.897	0.137	0.152	0.031	0.122	0.468	0.358
H8	2.217	0.323	0.393	0.437	1.215	0.917	0.363
H9	1.828	0.487	0.494	0.228	1.796	0.967	0.349
H10	1.859	0.572	0.417	0.336	2.317	1.100	0.411
H11	1.973	0.572	0.530	0.510	2.123	1.142	0.371
H12	1.953	0.461	0.633	0.161	1.532	0.948	0.340
Mean (explants)	1.966	0.483	0.494	0.334	1.797	1.015	0.151
SEM	0.068	0.046	0.043	0.064	0.198		

[a] = not represented in figure 6.1.

Mucus secreted (μg/ml)

Control filtrates: Ileum explants

	A1	A2	A4	A11	A12
H8	0.929	-0.621	2.995	2.535	1.139
H9	-1.003	1.152	0.389	0.319	0.023
H10	-0.776	-2.728	3.050	1.338	1.091
H11	1.035	0.316	3.605	4.717	2.682
H12	-0.261	-0.060	0.768	2.342	0.806

Control filtrates: Colon explants

	A1	A2	A4	A11	A12
H8	-0.145	0.170	0.292	0.693	0.150
H9	-0.363	-0.587	0.109	0.348	-0.003
H10	-0.547	-2.769	0.247	0.613	0.068
H11	-0.748	-3.526	0.279	0.575	0.165
H12	-0.987	1.779	0.369	0.402	0.324

Mucus secreted (μg/ml)

Ligation/no mucus filtrates: Ileum explants

	A3	A5	A6	A10	H2
H8	1.611	2.107	1.171	0.543	1.589
H9	0.241	2.993	0.543	0.027	0.688
H10	0.437	2.108	0.272	0.263	1.119
H11	0.968	2.802	1.555	1.625	3.945
H12	0.033	2.297	0.712	0.192	1.532

Ligation/no mucus filtrates: Colon explants

	A3	A5	A6	A10	H2
H8	0.194	3.200	0.432	0.053	0.984
H9	0.011	5.380	0.594	0.101	0.983
H10	-0.040	3.969	0.925	0.141	0.995
H11	0.171	3.459	0.691	0.358	1.480
H12	-0.030	2.972	0.999	0.198	0.912

Mucus secreted (μg/ml)

Ligation/mucus filtrates: Ileum explants

	A7	A8	H5	H6	H7
H8	2.993	0.988	1.979	0.814	1.584
H9	-0.207	0.037	0.127	0.263	0.533
H10	0.581	0.313	0.468	0.240	0.448
H11	3.732	1.487	2.477	1.658	2.208
H12	0.851	0.058	0.967	0.489	1.153

Ligation/mucus filtrates: Colon explants

	A7	A8	H5	H6	H7
H8	0.320	0.186	0.241	0.405	1.093
H9	-0.069	0.351	0.342	0.196	1.675
H10	-0.038	0.436	0.265	0.304	2.195
H11	0.076	0.436	0.378	0.479	2.002
H12	0.056	0.324	0.481	0.130	1.411

Explant weights (mg)

Control filtrates: Ileum explants

	A1	A2	A4	A11	A12
H8	22	22	21	21	19
H9	30	25	27	20	27
H10	25	19	29	31	21
H11	25	20	20	24	27
H12	24	18	23	23	22

Control filtrates: Colon explants

	A1	A2	A4	A11	A12
H8	28	22	28	47	24
H9	16	22	26	30	22
H10	27	23	30	27	25
H11	34	42	29	26	32
H12	26	25	27	31	22

Explant weights (mg)

Ligation/no vessel filtrates: Ileum explants

	A3	A5	A6	A10	H2
H8	25	23	24	28	21
H9	24	31	28	27	27
H10	23	20	26	32	23
H11	30	22	26	27	22
H12	26	23	27	28	25

Ligation/no vessel filtrates: Colon explants

	A3	A5	A6	A10	H2
H8	30	23	24	24	26
H9	25	28	29	21	25
H10	25	27	24	28	26
H11	31	32	27	33	27
H12	23	30	30	24	23

Explant weights (mg)

Ligation/vessel filtrates: Ileum explants

	A7	A8	H5	H6	H7
H8	32	22	24	23	29
H9	23	28	24	23	31
H10	29	25	13	20	29
H11	23	27	24	19	24
H12	20	21	24	23	19

Ligation/vessel filtrates: Colon explants

	A7	A8	H5	H6	H7
H8	28	28	26	28	26
H9	26	23	27	29	22
H10	29	22	24	22	23
H11	29	26	29	29	30
H12	31	27	31	26	30

Mucus secreted (ng/mg explant tissue)

Control filtrates: Ileum explants

	A1	A2	A4	A11	A12	Mean	SEM
H8	42.23	-28.21	142.62	120.71	59.92	67.45	30.27
H9	-33.45	46.07	14.41	15.96	0.84	8.77	12.88
H10	-31.06	-143.60	105.18	43.18	51.96	5.13	43.06
H11	41.40	15.78	180.25	196.52	99.34	106.66	36.10
H12	-10.85	-3.33	33.38	101.85	36.64	31.54	19.98
Mean	1.65	-22.66	95.17	95.64	49.74	43.91	14.70
SEM	16.86	32.58	31.57	31.58	16.02		

Control filtrates: Colon explants

	A1	A2	A4	A11	A12	Mean	SEM
H8	-5.17	7.74	10.41	14.75	6.26	6.80	3.32
H9	-22.67	-26.69	4.21	11.60	-0.16	-6.74	7.59
H10	-20.24	-120.40	8.23	22.70	2.73	-21.40	25.70
H11	-22.01	-83.96	9.63	22.10	5.17	-13.81	18.96
H12	-37.98	71.16	13.68	12.98	14.74	14.92	17.27
Mean	-21.62	-30.43	9.23	16.83	5.75	-4.05	7.32
SEM	5.20	33.73	1.54	2.33	2.50		

Mucus secreted (ng/mg explant tissue)

Ligation/no vessel filtrates: Ileum explants

	A3	A5	A6	A10	H2	Mean	SEM
H8	64.42	91.62	48.79	19.38	75.66	59.97	12.33
H9	10.03	96.55	19.40	1.00	25.47	30.49	17.03
H10	18.98	105.41	10.46	8.22	48.66	38.35	18.25
H11	32.27	127.34	59.79	60.19	179.34	91.79	26.92
H12	1.28	99.88	26.36	6.87	61.28	39.14	18.46
Mean	25.40	104.16	32.96	17.13	78.08	51.95	9.05
SEM	11.02	6.21	9.23	10.69	26.62		

Ligation/no vessel filtrates: Colon explants

	A3	A5	A6	A10	H2	Mean	SEM
H8	6.45	139.14	18.00	2.19	37.85	40.73	5.14
H9	0.46	192.15	20.49	4.80	39.30	51.44	7.83
H10	-1.60	147.00	38.55	5.05	38.26	45.45	23.36
H11	5.53	108.11	25.60	10.84	54.81	40.98	16.23
H12	-1.29	99.06	33.30	8.27	39.65	35.80	13.24
Mean	1.91	137.09	27.19	6.23	41.97	42.88*	10.55
SEM	1.17	16.46	3.86	1.50	3.23		

* = significantly different from control ($P < 0.05$)

Mucus secreted (ng/mg explant tissue)

Ligation/vessel filtrates: Ileum explants

	A7	A8	H5	H6	H7	Mean	SEM
H8	93.54	44.91	82.47	35.38	54.63	62.19	11.11
H9	-9.02	1.33	5.28	11.43	17.18	5.24	4.47
H10	20.04	12.54	35.99	11.98	15.46	19.20	4.43
H11	162.26	55.09	103.23	87.25	91.98	99.96	17.51
H12	42.57	2.78	40.29	21.26	60.68	33.52	9.90
Mean	61.88	23.33	53.45	33.46	47.99	44.02	8.14
SEM	30.19	11.17	17.49	14.13	14.40		

Ligation/mucus filtrates: Colon explants

	A7	A8	H5	H6	H7	Mean	SEM
H8	11.42	6.64	9.29	14.47	42.04	16.77	6.45
H9	-2.65	15.24	12.68	6.76	76.13	21.63	13.97
H10	-1.31	19.80	11.06	13.83	95.45	27.77	17.27
H11	2.63	16.75	13.04	16.50	66.73	23.13	11.20
H12	1.82	12.00	15.53	4.98	47.03	16.27	8.07
Mean	2.38	14.09	12.32	11.31	65.47	21.11	4.99
SEM	2.46	2.25	1.04	2.28	9.75		

ANALYSIS OF VARIANCE TABLES FOR CHAPTER 6

Ileum: Mucus secretion (ng/mg tissue)

Source	SS	df	MS	F
Main effects				
Group	1062	2	531	0.138
Explant	73226	4	18306	4.781
Interactions				
Group/exp	78733	8	9841	2.570
Error	229708	60	3828	
Total	362729	74		

Colon: Mucus secretion (ng/mg tissue)

Source	SS	df	MS	F	LSD
Main effects					
Group	27573	2	13786	7.504*	24.25
Explant	447	4	111	0.060	
Interactions					
Group/exp	33123	8	4140	2.253	
Error	110223	60	1837		
Total	171366	74			

* $P < 0.005$

APPENDIX D
DATA TABLES FOR CHAPTER 7 (RADIOACTIVITY)

Counts with background subtracted, and corrected for quenching by external standard ratio

Control filtrates:

	Counts in undiluted media		Calculated from 1:45 dilution		Explants		Counts in media/ total counts			
	Day 1	Day 4	Day 4	Day 5	Day 1	Day 4	Day 1	Day 4	Day 4 (dil)	Day 5
Explant:										
1	2781	3056	5159	7515	2295	3905	0.548	0.439	0.569	0.658
2	2797	2913	4102	7245	1454	2927	0.658	0.499	0.584	0.712
3	2732	2929	5582	7875	1981	3640	0.580	0.446	0.605	0.684
4	2826	3026	6154	8820	1444	3914	0.662	0.436	0.611	0.693
5	3886	3828	8831	13635	2720	5131	0.588	0.427	0.632	0.727
Mean	3004	3151	5966	9018	1979	3903	0.603	0.447	0.604	0.698
SEM	221	172	791	1185	246	356	0.023	0.013	0.011	0.012

Counts with background subtracted, and corrected for quenching by external standard ratio

Control filtrates with bethanachol:

	Counts in undiluted media		Calculated from 1:45 dilution		Explants		Counts in media/ total counts			
	Day 1	Day 4	Day 4	Day 5	Day 1	Day 4	Day 1	Day 4	Day 4 (dil)	Day 5
Explant:										
1	3083	3209	7626	11655	1215	2810	0.717	0.533	0.731	0.806
2	2866	3153	6578	8550	1086	2683	0.725	0.540	0.710	0.761
3	3083	2993	6023	9495	1376	3096	0.691	0.492	0.660	0.754
4	2748	2763	6835	9765	2524	4252	0.521	0.394	0.617	0.697
5	2273	2331	5395	6885	1290	2674	0.638	0.466	0.669	0.720
Mean	2811	2890	6491	9270	1498	3103	0.652	0.482	0.677	0.749
SEM	149	160	376	781	261	297	0.038	0.027	0.020	0.019

Counts with background subtracted, and corrected for quenching by external standard ratio

Ligation/no vessel, no mucus hypersecretion filtrates:

	Counts in undiluted media		Calculated from 1:45 dilution		Explants		Counts in media/ total counts			
	Day 1	Day 4	Day 4	Day 5	Day 1	Day 4	Day 1	Day 4	Day 4 (dil)	Day 5
Explant:										
1	1957	2126	6438	8595	1071	2631	0.646	0.447	0.710	0.766
2	2329	2470	5789	8145	1032	2588	0.693	0.488	0.691	0.759
3	2550	2612	7292	9630	1718	3393	0.597	0.435	0.682	0.739
4	2587	2828	6181	10080	1460	2888	0.639	0.495	0.682	0.777
5	2974	2983	7236	14040	2998	6651	0.498	0.310	0.521	0.679
Mean	2480	2604	6587	10098	1656	3630	0.600	0.418	0.645	0.736
SEM	167	148	295	1045	359	769	0.033	0.033	0.034	0.017

Counts with background subtracted, and corrected for quenching by external standard ratio

Ligation with mucus hypersecretion filtrates:

	Counts in undiluted media		Calculated from 1:45 dilution		Explants		Counts in media/ total counts			
	Day 1	Day 4	Day 4	Day 5	Day 1	Day 4	Day 1	Day 4	Day 4 (dil)	Day 5
Explant:										
1	3402	3693	7278	9540	1281	2699	0.726	0.578	0.729	0.779
2	3549	3938	7785	12465	1243	2653	0.741	0.597	0.746	0.825
3	4018	4009	5649	9315	2548	3834	0.612	0.511	0.596	0.708
4	3285	3576	9298	12105	1144	2763	0.742	0.564	0.771	0.814
5	6562	7061	14349	19260	3519	6017	0.651	0.540	0.705	0.762
Mean	4163*	4455*	8872	12537	1947	3593	0.681	0.554*	0.712	0.777*
SEM	613	656	1488	1799	470	644	0.027	0.015	0.030	0.021

* = significantly different from control ($P < 0.05$)

ANALYSIS OF VARIANCE TABLES, CHAPTER 7 (RADIOACTIVITY)

Counts in undiluted media, day 1:

Source	SS	df	MS	F	LSD
Treatment	8035274	3	2678425	5.35*	976
Blocks	3469493	4	867373	1.73	
Error	6013300	12	501108		
Total	17518067	19			

Counts in undiluted media, day 4:

Source	SS	df	MS	F	LSD
Treatment	10038860	3	3346287	5.65*	1060
Blocks	3047190	4	761798	1.29	
Error	7103036	12	591920		
Total	20189087	19			

Calculated from 1:45 dilution, day 4:

Source	SS	df	MS	F
Treatment	25010161	3	8336720	2.56
Blocks	22349087	4	5587272	1.72
Error	39033051	12	3252754	
Total	86392299	19		

* $P < 0.05$

Calculated from 1:45 dilution, day 5:

Source	SS	df	MS	F
Treatment	38651074	3	12883691	2.16
Blocks	55272983	4	13818246	2.32
Error	71568158	12	5964013	
Total	1.7E+08	19		

Explants, day 1:

Source	SS	df	MS	F
Treatment	808756	3	269585	0.67
Blocks	4760451	4	1190113	2.97
Error	4800961	12	400080	
Total	10370168	19		

Explants, day 4:

Source	SS	df	MS	F
Treatment	1664090	3	554696	0.63
Blocks	13852704	4	3463176	3.93
Error	10566780	12	880565	
Total	26083573	19		

Counts in media/total counts, day 1:

Source	SS	df	MS	F
Treatment	0.0248	3	0.00827	2.14
Blocks	0.0278	4	0.00696	1.80
Error	0.0463	12	0.00386	
Total	0.0989	19		

Counts in media/total counts, day 4:

Source	SS	df	MS	F	LSD
Treatment	0.0454	3	0.0151	7.64*	0.061
Blocks	0.0204	4	0.0051	2.57	
Error	0.0237	12	0.0020		
Total	0.0895	19			

Counts in media/total counts, day 4, 1:45 dilution:

Source	SS	df	MS	F
Treatment	0.0315	3	0.01045	2.98
Blocks	0.0104	4	0.0026	0.74
Error	0.0422	12	0.0035	
Total	0.0840	19		

* $P < 0.05$

Counts in media/total counts, day 5, 1:45 dilution:

Source	SS	df	MS	F	LSD
Treatment	0.0177	3	0.00592	3.79*	0.054
Blocks	0.0057	4	0.00143	0.92	
Error	0.0187	12	0.00156		
Total	0.0421	19			

* $P < 0.05$

APPENDIX E
DATA TABLES FOR CHAPTER 7 (ELLA)

Mucus in media/filtrate, calculated from ELLA (μg/ml)

	Control	Bethanechol	Lig/no mucus	Ligation/mucus
No explant	3.153	3.022	-0.402	-0.672
1	3.648	3.578	0.481	4.851
2	2.998	3.656	0.295	3.972
3	2.911	3.301	0.327	3.586
4	2.864	2.852	0.545	4.516
5	3.105	2.699	0.369	3.044
Explant mean	3.105	3.217	0.403	3.994
SEM	0.142	0.191	0.047	0.322

Mucus Secreted (μg/ml)

	Control	Bethanechol	Lig/no mucus	Ligation/mucus
1	0.496	0.556	0.882	5.523
2	-0.154	0.634	0.697	4.644
3	-0.241	0.279	0.729	4.258
4	-0.289	-0.170	0.947	5.189
5	-0.047	-0.323	0.771	3.717
Mean	-0.047	0.196	0.805*	4.666**
SEM	0.142	0.191	0.047	0.322

* = significantly different from control ($P < 0.05$)

**= significantly different from control ($P < 0.002$)

ANALYSIS OF VARIANCE TABLE FOR CHAPTER 7 (ELLA)

Source	SS	df	MS	F	$LSD_{.05}$	$LSD_{.002}$
Treatment	72.834	3	24.278	166.847*	0.526	0.948
Blocks	1.508	4	0.377	2.591		
Error	1.746	12	0.146			
Total	76.088	19				

* P < 0.001

APPENDIX F
DATA TABLES FOR CHAPTER 8 (RADIOACTIVITY)

Counts in media/filtrate : Total counts

		Untreated		Precipitated		Trypsin		Chymotrypsin		Serum	
		Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus
H15	1	0.682	0.785	0.739	0.779	0.712	0.746	0.723	0.767	0.771	0.790
	2	0.728	0.700	0.752	0.757	0.688	0.681	0.723	0.710	0.768	0.819
	3	0.657	0.666	0.702	0.687	0.614	0.642	0.656	0.683	0.716	0.747
H16	1	0.650	0.658	0.771	0.803	0.695	0.697	0.682	0.676	0.760	0.765
	2	0.662	0.772	0.768	0.826	0.710	0.728	0.710	0.693	0.800	0.815
	3	0.703	0.750	0.761	0.783	0.739	0.743	0.741	0.730	0.773	0.794
Mean		0.680	0.722*	0.749	0.773	0.693	0.706	0.706	0.710	0.765	0.788
SEM		0.012	0.022	0.011	0.020	0.017	0.017	0.013	0.014	0.011	0.012

* = significantly different from control ($P < 0.05$)

Radioactivity in ammonium sulfate-precipitated material

Average of triplicate counts with background subtracted

		Untreated		Precipitated		Trypsin		Chymotrypsin		Serum	
		Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus
H15	1	48.67	40.33	33.67	39.33	43.00	43.00	27.33	42.67	30.33	48.33
	2	34.33	34.33	21.67	47.67	36.67	47.33	34.33	52.00	64.00	86.67
	3	22.33	40.67	29.00	35.67	28.67	25.00	33.67	27.33	41.67	44.00
H16	1	21.67	32.67	33.67	46.33	30.00	45.67	44.33	46.67	36.00	39.00
	2	39.67	46.67	26.33	45.67	31.00	30.67	32.00	31.00	68.33	80.67
	3	26.00	39.67	20.33	31.33	49.00	48.33	41.00	34.33	56.33	75.33
Mean		32.11	39.06	27.44	41.00*	36.39	40.00	35.44	39.00	49.44	62.33*
SEM		4.39	2.05	2.35	2.70	3.33	3.99	2.53	3.93	6.38	8.51

* = significantly different from control ($P < 0.05$)

ANALYSIS OF VARIANCE TABLES, CHAPTER 8 (RADIOACTIVITY)

Proportion of counts in media/filtrate vs. total:

Source	SS	df	MS	F	$LSD_{.05}$
Treatment	0.073	9	0.0081	13.03*	0.029
Blocks	0.042	5	0.0084	13.47	
Error	0.028	45	0.0006		
Total	0.143	59			

* P < 0.001

Counts in media/filtrate precipitated with ammonium sulfate:

Source	SS	df	MS	F	$LSD_{.05}$
Treatment	5064	9	562.70	5.19*	12.15
Blocks	1072	5	214.32	1.98	
Error	4877	45	108.37		
Total	11013	59			

* P < 0.001

APPENDIX G
DATA TABLES FOR CHAPTER 8, PART A (ELLA)

Mucus in media/filtrate, calculated from ELLA (μg/ml)

	Untreated		Precipitated		Trypsin		Chymotrypsin		Serum	
	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus
H15 blank	1.927	0.148	1.610	0.405	1.809	0.423	1.678	0.321	0.049	0.507
1	2.076	2.943	1.485	4.514	1.927	2.718	1.902	2.613	0.049	0.555
2	1.746	2.565	1.759	3.662	1.790	2.853	1.865	2.235	-0.076	0.609
3	1.703	2.265	1.678	2.757	1.858	1.815	1.678	1.899	-0.101	0.891
H16 blank	1.112	0.069	0.992	-0.076	1.178	-0.003	1.288	-0.097	-0.077	0.008
1	1.264	2.012	1.312	2.521	1.421	2.063	1.436	1.889	0.063	0.023
2	1.283	1.520	1.312	2.657	1.312	1.604	1.493	1.876	-0.018	0.071
3	1.245	1.599	1.374	2.371	1.379	1.841	1.135	1.542	0.020	0.145
Means:										
Blanks	1.519	0.039	1.301	0.164	1.494	0.210	1.483	0.112	0.055	0.265
Explants	1.553	2.150	1.486	3.080	1.614	2.048	1.584	2.009	-0.025	0.454
SEM:										
Blanks	0.407	0.108	0.308	0.241	0.315	0.213	0.195	0.209	0.014	0.250
Explants	0.139	0.226	0.078	0.341	0.111	0.210	0.118	0.150	0.027	0.144

Mucus Secreted (μg/ml)

		Untreated		Precipitated		Trypsin		Chymotrypsin		Serum	
		Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus
H15	1	0.149	2.795	-0.124	4.109	0.118	2.291	0.224	2.291	0	0.048
	2	-0.180	2.417	0.149	3.257	-0.019	2.429	0.187	1.913	-0.124	0.102
	3	-0.224	2.117	0.068	2.351	0.050	1.391	0	1.577	-0.149	0.384
H16	1	0.153	2.080	0.319	2.598	0.243	2.065	0.148	1.986	-0.014	0.015
	2	0.172	1.588	0.319	2.733	0.133	1.606	0.205	1.973	-0.095	0.064
	3	0.133	1.667	0.381	2.448	0.200	1.843	-0.153	1.639	-0.057	0.138
Mean		0.034	2.111*	0.186	2.916*	0.121	1.938*	0.102	1.897*	-0.073	0.125
SEM		0.075	0.186	0.079	0.272	0.039	0.163	0.061	0.106	0.024	0.055

* = significantly different from control ($P < 0.05$)

Source	SS	df	MS	F	$LSD_{.05}$
Treatment	69.85	9	7.76	91.95*	0.339
Blocks	1.20	5	0.24	2.85	
Error	3.80	45	0.084		
Total	74.85	59			

* P < 0.001

APPENDIX H
DATA TABLES FOR CHAPTER 8, PART B (ELLA)

Mucus in media/filtrate, calculated from ELLA (μg/ml)

	Untreated-Filtrate		Heat-Filtrate		Acid-Filtrate		Untreated-Serum		Heat-Serum	
	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus
Blank	1.552	-0.433	1.941	-0.443	1.687	-0.357	-0.610	-0.328	-0.613	-0.279
1	1.434	0.975	1.671	-0.327	1.459	-0.414	-0.502	-0.316	-0.567	-0.321
2	1.401	1.055	1.754	-0.304	1.177	-0.388	-0.524	-0.244	-0.334	-0.112
3	1.629	1.085	2.613	-0.350	1.658	-0.304	-0.470	-0.391	-0.538	-0.280
4	0.894	0.643	1.691	-0.422	1.546	-0.445	-0.663	-0.391	-1.042	-0.716
5	1.588	0.997	2.186	-0.266	1.401	-0.239	-0.413	-0.348	-1.042	-0.716
Explant:										
Mean	1.389	0.951	1.983	-0.333	1.448	-0.358	-0.515	-0.338	-0.705	-0.429
SEM	0.131	0.080	0.183	0.026	0.081	0.038	0.042	0.028	0.143	0.122

Mucus Secreted (μg/ml)

	Untreated-Filtrate		Heat-Filtrate		Acid-Filtrate		Untreated-Serum		Heat-Serum	
	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus	Control	Mucus
1	-0.118	1.408	-0.270	0.116	-0.228	-0.057	0.107	0.011	0.046	-0.042
2	-0.152	1.488	-0.187	0.139	-0.511	-0.031	0.086	0.084	0.279	0.167
3	0.077	1.519	0.673	0.093	-0.029	0.053	0.139	-0.064	0.075	-0.001
4	-0.658	1.076	-0.249	0.021	-0.141	-0.088	-0.054	-0.064	-0.429	-0.437
5	0.035	1.431	0.245	0.177	-0.286	0.118	0.197	-0.020	-0.429	-0.437
Mean	-0.163	1.384	0.042	0.110	-0.239	-0.001	0.095	-0.010	-0.091	-0.150
SEM	0.131	0.080	0.183	0.026	0.081	0.038	0.042	0.028	0.143	0.122

ANALYSIS OF VARIANCE TABLE FOR CHAPTER 8, PART B (ELLA)

Source	SS	df	MS	F	$LSD_{.05}$	$LSD_{.002}$
Treatment	9.79	9	1.09	27.61	254	417
Blocks	0.66	4	0.16	4.17		
Error	1.42	36	0.04			
Total	11.86	49				

REFERENCE LIST

1) Ahmad, A., C. H. Wang, M. Korenaga, R. G. Bell, and L. S. Adams. 1990. Synergistic interaction between immune serum and thoracic duct cells in the adoptive transfer of rapid expulsion of *Trichinella spiralis* in adult rats. Exp Parasitol 71:90-99.

2) Alarcón de la Lastra, C., A. López, and V. Motilva. 1993. Gastroprotection and prostaglandin E_2 generation in rats by flavonoids of *Dittrichia viscosa*. Planta Med 59:497-501.

3) Alarcón de la Lastra, C., M. J. Martin, and V. Motilva. 1994. Antiulcer and gastroprotective effects of quercetin: a gross and histologic study. Pharmacology 48:56-62.

4) Allen, A., W. J. Cunliffe, J. P. Pearson, L. A. Sellers, and R. Ward. 1984. Studies on gastrointestinal mucus. Scand J Gastroent Suppl 93:101-113.

5) Allen, A., A. J. Leonard, and L. A. Sellers. 1988. The mucus barrier. Its role in gastroduodenal protection. J Clin Gastroenterol 10(Suppl 1):S93-S98.

6) Argenzio, R. A. 1985. Digestion, absorption, and metabolism. In: Dukes' Physiology of Domestic Animals, Tenth Edition. Eds. Swenson, M. J. and W. O. Reece. Ithaca: Cornell University Press. p.262-277.

7) Argenzio, R. A., M. Southworth, J. E. Lowe, and C. E. Stevens. 1977. Interrelationship of NA, HCO_3, and volatile fatty acid transport by equine large intestine. Am J Physiol 233 (Endocrinol Metab Gastrointest Physiol 2):E469-E478.

8) Arnold, J. W., G. R. Klimpel, and D. W. Niesel. 1993. Tumor necrosis factor (TNFα) regulates intestinal mucus production during salmonellosis. Cell Immunol 151:336-344.

9) Babkin, B. P. 1950. Secretory Mechanism of the Digestive Glands. 2nd Ed. New York: Hoeber.

10) Barthold, S. W., G. L. Coleman, R. O. Jacoby, E. M. Livstone, and A. M. Jonas. 1978. Transmissible murine colonic hyperplasia. Vet Pathol 15:223-236.

11) Bensley, B. A. 1938. Practical Anatomy of the Rabbit, 6th ed. Philadelphia: P. Blaciston's Son + Co., Inc.

12) Biol, M. C., A. Martin, M. Richard, and P. Louisot. 1987. Developmental changes in intestinal glycosyl-transferase activities. Pediatr Res 22:250-256.

13) Black, B. L. and F. Moog. 1977. Goblet cells in embryonic intestine: accelerated differentiation in culture. Science 197:368-370.

14) Bradbury, J. E., J. W. Black, and J. H. Wyllie. 1980. Stimulation of mucus output from rat colon in vivo. Eur J Pharmacol 68:417-25.

15) Bradley, H. C. 1933. Inhibition of pepsin by mucin. J Biol Chem 100:xx.

16) Bradley, H. C. and M. Hodges. 1934. The effect of mucin and mucinoids on peptic digestion. J Lab Clin Med 20:165-169.

17) Bucher, R. 1932. Das Wesen der Schutzwirkung des Magenschleims. Deutsche Zeitschr Chir 236:515.

18) Carlisle, M. S., D. D. McGregor, and J. A. Appleton. 1990. The role of mucus in antibody-mediated rapid expulsion of *Trichinella spiralis* in suckling rats. Immunol 70:126-132.

19) Carlson, D. M. 1977. Mucous glycoproteins. Mod Probl Paediat 19:1-10.

20) Carman, R. J. and R. H. Evans. 1984. Experimental and spontaneous clostridial enteropathies of laboratory and free living lagamorphs. Lab Anim Sci 34:443-452.

21) Cepinskas, G., R. D. Specian, and P. R. Kvietys. 1993. Adaptive cytoprotection in the small intestine: role of mucus. Am J Physiol 264 (Gastrointest Liver Physiol 27):G921-G927.

22) Chadee, K., K. Keller, J. Forstner, D. J. Innes, and J. I. Ravdin. 1991. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterol 100:986-997.

23) Chambraud, L., A. Bernadac, J. P. Gorvel, and S. Maroux. 1989. Renewal of goblet cell mucus granules during the cell migration along the crypt-villus axis in rabbit jejunum: an immunolabeling study. Biol Cell 65:151-162.

24) Chen, C. C., M. Baylor, and D. M. Bass. 1993. Murine intestinal mucins inhibit rotavirus infection. Gastroenterology 105:84-92.

25) Chiu, P. J., A. Barnett, M. Siegel, A. D. Brown, and C. Gerhart. 1988. Protective action of SCH12223 against experimentally induced gastric and intestinal lesions. J Pharmacol Exp Ther 246:578-584.

26) Clarke, L. L. and R. A. Argenzio. 1990. NaCl transport across equine proximal colon and the effect of endogenous prostanoids. Am J Physiol 259 (Gastrointest Liver Physiol 22):G62-G69.

27) Cohan, V. L., A. L. Scott, C. A. Dinarello, and R. A. Prendergast. 1991. Interleukin-1 is a mucus secretagogue. Cell Immunol 136:425-434.

28) Condor, G. A., L. F. Mayberry, J. R. Bristol, G. A. Castro, B. L. Lee, D. D. Kratzer, S. D. Folz, and D. L. Rector. 1987. Effects of PGE_1 or PGE_2 and/or acetazolamide on expulsion of *Nippostrongylus braziliensis* from rats. Prostaglandins 34:817-827.

29) Corfield, A. P., A. D. Casey, S. A. Wagner, J. Eldridge, M. Cox, C. D. Corfield, and J. R. Clamp. 1987. Selection of radioactive precursors for metabolic labelling of mucus glycoproteins. Biochem Soc Trans 17:1037-1038.

30) Cramer, W. and A. N. Kingsbury. 1924. Local and general deficiencies against infections, and the effect on them of vitamin-deficiency. Brit J Exp Path 5:300.

31) Crampton, J. R. 1988. Gastroduodenal mucus and bicarbonate: The defensive zone. Q J Med 252:269-272.

32) Crowther, R. S., N. W. Roomi, R. E. F. Fahim, and J. F. Forstner. 1987. *Vibrio cholerae* metalloproteinase degrades intestinal mucin and facilitates enterotoxin-induced secretion from rat intestine. Biochim Biophys Acta 924:393-402.

33) Demeš, P., F. F. Pindak, D. J. Wells, and W. A. Gardner Jr. 1989. Adherence and surface properties of *Tritrichomonas mobilensis*, an intestinal parasite of the squirrel monkey. Parasitol Res 75:589-594.

34) Desai, M. A., C. V. Nicholas, and P. Vadgama. 1991. Electrochemical determination of the permeability of porcine mucus to model solute compounds. J Pharm Pharmacol 43:124-127.

35) Desai, M. A. and P. M. Vadgama. 1993. An *in vitro* study of enhanced H^+ diffusion by urease action on urea. Scand J Gastroenterol 28:915-919.

36) Dobson, C. 1967. Changes in the protein content of the serum and intestinal mucus of sheep with reference to the histology of the gut and immunological response to *Oesophagostomum columbianum* infections. Parasitol 57:201-219.

37) Drumm, B., A. W. Neumann, Z. Policova, and P. M. Sherman. 1989. Bacterial cell surface hydrophobicity properties in the mediation of *in vitro* adhesion by the rabbit enteric pathogen *Escherichia coli* strain RDEC-1. J Clin Invest 84:1588-1594.

38) Duthie, E. S. 1933. Mucus formation in goblet cells. Proc Roy Soc London Series B 113:459-463.

39) Englard, S. and S. Seifter. 1990. Precipitation techniques. Methods Enzymol 182:285-300.

40) Farack, U. M., J. Reiter, M. Gross, L. Moroder, E. Wünsch, and K. Loeschke. 1987. Influence of vasoactive intestinal peptide, secretin, and Ala^4, Val^5-secretin on the net movements of electrolytes, fluid, and mucus in the rat colon *in vivo*. Scand J Gastroenterol Suppl 139:32-36.

41) Feizi, T. 1982. Antigenicities of mucins - their relevance to tumor associated and stage specific embryonic antigens. Adv Exp Med Biol 144:29-37.

42) Ferrero, R. L. and A. Lee. 1988. Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. J Gen Microbiol 134:53-59.

43) Filipe, M. I., A. Sandey, and J. Ma. 1988. Intestinal mucin antigens in ulcerative colitis and their relationship with malignancy. Hum Pathol 19:671-681.

44) Florey, H. 1930. The secretion of mucus by the colon. Brit J Exp Path 11:348-361.

45) Florey, H. 1931. The mechanism of goblet cell secretion in the mammal; the effects of cyanide. Brit J Exp Path 12:301-305.

46) Florey, H. W. 1933. Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa. J Path Bacteriol 37:283-289.

47) Florey, H. 1955. Mucin and the protection of the body. Proc Roy Soc London Series B 143:147-158.

48) Forlong, C., C. Tasman-Jones, L. Thomsen, and J. Clearwater. 1990. The Na^+/H^+ exchange and H^+ diffusion properties of human postmortem mucus: A comparison of gastric antral, gastric body, jejunal, and ileal mucus. J Clin Gastroenterol 12(Suppl 1):S110-S115.

49) Forstner, J. F., N. W. Roomi, R. E. F. Fahim, and G. G. Forstner. 1981. Cholera toxin stimulates secretion of immunoreactive intestinal mucin. Am J Physiol 240 (Gastrointest Liver Physiol 3):G10-G16.

50) Forstner, G., M. Shih, and B. Lukie. 1973. Cyclic AMP and intestinal glycoprotein synthesis: The effect of ß-adrenergic agents, theophylline, and dibutyryl cyclic AMP. Can J Physiol Pharmacol 51:122-129.

51) Forstner, J. F. 1978. Intestinal mucins in health and disease. Digestion 17:234-263.

52) Foster, H. L. 1980. Gnotobiology. In: The Laboratory Rat Volume II Research Applications Ed. Baker, H. J., J. R. Lindsey, and S. H. Weisbroth. New York:Academic Press, p.43-59.

53) Frase, L. L., A. D. Strickland, G. W. Kachel, and G. J. Krejs. 1985. Enhanced glucose absorption in the jejunum of patients with cystic fibrosis. Gastroenterol 88:478-484.

54) Frick, L. P. and J. E. Ackert. 1948. Further studies on duodenal mucus as a factor in age resistance of chickens to parasitism. J Parasit 34:192-206.

55) Fuller, C. E., R. P. Davies, G. T. Williams, and E. D. Williams. 1990. Crypt restricted heterogeneity of goblet cell mucus glycoprotein in histologically normal human colonic mucosa: a potential marker of somatic mutation. Br J Cancer 61:382-384.

56) Galen. 1968. On the Usefulness of the Parts of the Body. Translated by M. T. May. Ithaca: Cornell Univ Press.

57) Gault, M. J., F. D. Gillin, and A. J. Zenian. 1987. *Giardia lamblia*: stimulation of growth by human intestinal mucus and epithelial cells in serumfree medium. Exp Parasitol 64:29-37.

58) Gibson, L. E., W. J. Matthews, P. T. Minihan, and J. A. Patti. 1971. Relating mucus, calcium and sweat in a new concept of cystic fibrosis. Pediatrics 48:695-710.

59) Glass, G. B. J. 1953. Gastric mucin and its constituents: Physico-chemical characteristics, cellular origin, and physiological significance. Gastroenterol 23:636-658.

60) Goldstein, I. J. and R. D. Poretz. 1986. Isolation, physicochemical characterization, and charbohydrate-binding specificity of lectins. In: The Lectins. Ed. Leiner, I. E., N. Sharon, and I. J. Goldstein, Orlando: Academic Press. p. 35-250.

61) Goldsworthy, N. E. and H. Florey. 1930. Some properties of mucus, with special reference to its antibacterial functions. Brit J Exp Path 11:192-208.

62) Gong, D., B. Turner, K. R. Bhaskar, and J. T. Lamont. 1990. Lipid binding to gastric mucin: protective effect against oxygen radicals. Am J Physiol 259 (Gastrointest Liver Physiol 22):G681-G686.

63) Gorelick, F. S. and J. D. Jamieson. 1987. Structure-function relationship of the pancreas. In Physiology of the Gastrointestinal Tract, 2nd ed Ed. L. R. Johnson, New York: Raven Press. p. 1089-1108.

64) Greenham, L. W. 1962. Some preliminary observations on rabbit mucoid enteritis. Vet Rec 74:79-84.

65) Greenwood, B. and M. Mantle. 1992. Mucin and protein release in the rabbit jejunum: effects of bethanechol and vagal nerve stimulation. Gastroenterology 103:496-505.

66) Gruber, P., A. Rubinstein, V. H. K. Li, P. Bass, and J. R. Robinson. 1987. Gastric emptying of nondigestible solids in the fasted dog. J Pharmaceut Sci 76:117-122.

67) Gupta, B. L. 1989. The relationship of mucoid substances and ion and water transport, with new data on intestinal goblet cells and a model for gastric secretion. Symp Soc Exp Biol 43:81-110.

68) Guth, D. and W. von Engelhardt. 1989. Is gastrointestinal mucus an ion-selective barrier? Symp Soc Exp Biol 43:117-121.

69) Han, V., J. Resau, R. Predergast, A. Scott, and D. A. Levy. 1987. Interleukin-1 induces mucus secretion from mouse intestinal explants. Int Archs Allergy appl Immun 82:364-365.

70) Harkema, J. R. and J. A. Hotchkiss. 1991. *In vivo* effects of endotoxin on nasal epithelial mucosubstances: quantitative histochemistry. Exp Lung Res 17:743-761

71) Harlow, E. and D. Lane. 1988. Antibodies A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratories.

72) Hartiala, K. and M. I. Grossman. 1951. Studies on chemical and physical changes in duodenal mucus. J Biol Chem 195:251-256.

73) Hattori, T. and N. Arizono. 1988. Cell kinetics and secretion of mucus in the gastrointestinal mucosa, and their diurnal rhythm. J Clin Gastrenterol 10 (Suppl 1):S1-S6.

74) Heatley, N. G. 1956. Does mucin inhibit the action of pepsin? Quart J Exp Physiol 41:25-30.

75) Heatley, N. G. 1959. Mucosubstance as a barrier to diffusion. Gastroenterol 37:313-317.

76) Heidenhain, R. 1878. Ueber die Pepsinbildung in den Pylorusdrüsen. Archiv für Physiologie (Pflügers) 18:169-171.

77) Hill, B. D., D. A. Blewett, A. M. Dawson, and S. Wright. 1991. *Cryptosporidium parvum*: Investigation of sporozoite excystation *in vivo* and the association of merozoites with intestinal mucus. Res Vet Sci 51:264-267.

78) Hogben, C. A. M., D. J. Tocco, B. R. Brodie, and L. S. Schanker. 1959. On the mechanism of intestinal absorption of drugs. J Pharm Exp Ther 125:275-282.

79) Hollander, F. 1954. The two-component mucus barrier. Archs Intern Med 93:107-129.

80) Hollander, F., B. P. Sonnenblick, and H. A. Sober. 1947. Experimental impairment of the gastric mucous barrier in dogs. J Natl Cancer Inst 7:361-364.

81) Hollander, F., J. Stein, and F. U. Lauber. 1946. The consistency, opacity, and columnar cell content of gastric mucus secreted under the influence of several mild irritants. Gastroenterol 6:576-595.

82) Hoskins, L. C., M. Agustines, W. B. McKee, E. T. Boulding, M. Kriaris, and G. Niedermeyer. 1985. Mucin degradation in human colon ecosystems. J Clin Invest 75:944-953.

83) Huet, C., C. Sahuquillo-Merino, E. Coudrier, and D. Louvard. 1987. Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. J Cell Biol 105:345-357.

84) Ichikawa, M., A. Ichikawa, and S. Kidokoro. 1987. Secretory process of mucus-secreting cells in mouse colonic mucosa studied by rapid freezing and freeze-substitution. J Electron Microsc Tokyo 36:117-127.

85) Ichikawa, T., K. Ishihara, Y. Komuro, Y. Kojima, K. Saigenji, and K. Hotta. 1994. Effects of the new histamine H_2 receptor antagonist, FRG-8813, on gastric mucin in rats with or without acidified ethanol-induced gastric damage. Life Sci 54:PL159-PL164.

86) Ichikawa, T., K. Ishihara, K. Saigenji, and K. Hotta. 1993. Stimulation of mucus glycoprotein biosynthesis in rat gastric mucosa by gastrin. Biochem Pharmacol 46:1551-1557.

87) Ichikawa, T., K. Ishihara, K. Saigenji, and K. Hotta. 1994. Effects of acid-inhibitory antiulcer drugs on mucin biosynthesis in the rat stomach. Eur J Pharmacol 251:107-111.

88) Ishikawa, N., Y. Horii, T. Oinuma, T. Suganuma, and Y. Nawa. 1994. Goblet cell mucins as the selective barrier for the intestinal helminths: T-cell-independent alteration of goblet cell mucins by immunologically 'damaged' *Nippostrongylus brasiliensis* worms and its significance on the challenge infection with homologous and heterologous parasites. Immunology 81:480-486.

89) Itagaki, S., C. J. Perfumo, M. A. Petruccelli, and K. Doi. 1994. Lectin histochemical changes of colon goblet cell mucin in rabbit mucoid enteropathy. Lab Anim Sci 44:82-84.

90) Ito, S. 1987. Functional gastric morphology. In Physiology of the Gastrointestinal Tract, 2nd ed Ed. L. R. Johnson, New York: Raven Press. p. 817-851.

91) Janowitz, H. D. and F. Hollander. 1951. Some properties of cell-free gastric mucus. Fed Proc 10:70.

92) Janowitz, H. D., F. Hollander, and C. Jackson. 1951. Stimulation of cell-free gastric mucus by the topical application of acetylcholine. Proc Soc Exp Biol Med 76:578-580.

93) Jarry, A., D. Merlin, A. Velcich, U. Hopfer, L. H. Augenlicht, and C. L. Laboisse. 1994. Interferon-γ modulates cAMP-induced mucin exocytosis without affecting mucin gene expression in a human colonic goblet cell line. Eur J Pharmacol 267:95-103.

94) Jass, J. R., K. Sugihara, and S. B. Love. 1988. Basis of sialic acid heterogeneity in ulcerative colitis. J Clin Pathol 41:388-392.

95) Jeynes, B. J. and G. G. Altman. 1978. Light and scanning electron microscopic observations of the effects of sublethal doses of methotrexate on the rat small intestine. Anat Rec 191:1-18.

96) Jubb, K. V. F., P. C. Kennedy, and N. Palmer. 1985. Pathology of Domestic Animals 3rd ed, Vol 2. New York: Academic Press.

97) Karlsson, J., A. Wikman, and P. Artursson. 1993. The mucus layer as a barrier to drug absorption in monolayers of human intestinal epithelial HT29-H goblet cells. Int J Pharm 99:209-218.

98) Kaufman, J. 1908. Lack of gastric mucus (amyxorrhea gastrica) and its relation to hyperacidity and gastric ulcer. Am J Med Sci 135:207-214.

99) Kaura, R., A. Allen, and B. H. Hirst. 1980. Mucus in the gastric juice of cats during pentagastrin and secretin infusions. The viscosity in relation to glycoprotein structure and concentration. Biochem Soc Trans 8:52-53.

100) Kemper, A. C. and R. D. Specian. 1991. Rat small intestinal mucins: a quantitative analysis. Anat Rec 229:219-226.

101) Kennedy, M. J., D. K. Rosnick, R. G. Ulrick, and R. J. Yancey Jr. 1988. Association of *Treponema hyodysenteriae* with porcine intestinal mucosa. J Gen Microbiol 134:1565-1576.

102) Kennedy, M. J., P. A. Volz, C. A. Edwards, and R. J. Yancey. 1987. Mechanisms of association of *Candida albicans* with intestinal mucosa. J Med Microbiol 24:333-341.

103) Kimambo, A. E. and J. C. MacRae. 1988. Measurement *in vitro* of a larval migration inhibitory factor in gastrointestinal mucus of sheep made resistant to the roundworm *Trichostrongylus colubriformis*. Vet Parasitol 28:213-222.

104) Koga, K., Y. Nonaka, T. Kawai, T. Seki, H. Kon, M. Tsurui, H. Ikeda, Y. Harada, T Saito, and S. Ashizawa. 1989. Changes in levels of mucosal glycoprotein on cysteamine-induced duodenal ulcers in rats. Scand J Gastroenterol Suppl 162:120-123.

105) Kraus, A. L., S. H. Weisbroth, R. E. Flatt, and N. Brewer. 1984. Biology and diseases of rabbits. In: Laboratory Animal Medicine. Ed. Fox J. G., B. J. Cohen, & F. M. Loew, New York: Academic Press. p. 207-240.

106) Kudweis, M., Z. Lojda, J. Vitovec, and B. Koudela. 1989. Mucus synthesis in the goblet cells of the small intestine in experimental infection with the coccidium *Isospora suis* in piglets. Vet Med Praha 43:727-734.

107) Kudweis, M., Z. Lojda, J. Vitovec, and B. Koudela. 1990. Activity of mucosubstances and the goblet cell count in the large intestine of piglets infected with the coccidium, *Isospora suis*. Vet Med Praha 35:21-29.

108) Laburthe, M., C. Augeron, C. Rouyer-Fessard, I. Roumagnac, J.-J. Maoret, E. Grasset, and C. Laboisse. 1989. Functional VIP receptors in the human mucus-secreting colonic epithelial cell line CL.16E. Am J Physiol 256 (Gastrointest Liver Physiol 19):G443-G450.

109) Lake, A. M., K. J. Bloch, M. R. Neutra, and W. A. Walker. 1979. Intestinal goblet cell mucus release. II. *In vivo* stimulation by antigen in the immunized rat. J Immunol 122:834-837.

110) LaMont, J. T. and A. Ventola. 1977. Stimulation of colonic glycoprotein synthesis by dibutyryl cyclic AMP and theophylline. Gastroenterol 72:82-86.

111) Lee, D. L. and W. D. Biggs. 1990. Two- and three-dimensional locomotion of the nematode *Nippostrongylus brasiliensis*. Parasitol 101:301-308.

112) Leitch, G. J. 1988. Intestinal lumen and mucosal microclimate H^+ and NH_3 concentrations as factors in the etiology of experimental amebiasis. Am J Trop Med Hyg 38:480-486.

113) Lelkes, L. 1986. Overeating and microbial imbalance in the development of mucoid enteropathy in rabbits. J Appl Rabbit Res 9:148-151.

114) Lelkes, L. and C.-L. Chang. 1987. Microbial dysbiosis in rabbit mucoid enteropathy. Lab Anim Sci 37:757-764.

115) Lencer, W. I., F. D. Reinhart, and M. R. Neutra. 1990. Interaction of cholera toxin with cloned human goblet cells in monolayer culture. Am J Physiol 258 (Gastrointest Liver Physiol 21):G96-G102.

116) Lesuffleur, T., N. Porchet, J.-P. Aubert, D. Swallow, J. R. Gum, Y. S. Kim, F. X. Real, and A. Zweibaum. 1993. Differential expression ofthe human mucin genes *MUC1* to *MUC5* in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. J Cell Sci 106:771-783.

117) Lewin, M. R., S. H. El-Masri, and C. G. Clark. 1979. Effects of bile acids on mucus secretion in the dog colon. Eur Surg Res 11:392-399.

118) Lima, A. A. M., D. J. Innes, K. Chadee, D. M. Lyerly, T. D. Wilkins, and R. L. Guerrant. 1989. *Clostridium difficile* toxin A. Interactions with mucus and early sequential histopathologic effects in rabbit small intestine. Lab Invest 61:419-425.

119) Lindahl, M. and I. Carlstedt. 1990. Binding of K99 fimbriae of enterotoxigenic *Escherichia coli* to pig small intestinal mucin glycopeptides. J Gen Microbiol 136:1609-1614.

120) Lindstedt, G., S. Lindstedt, and B. E. Gustafsson. 1965. Mucus in the intestinal contents of germfree rats. J Exp Med 121:201-213.

121) Loeschke, K., T. Schmid, and U. M. Farack. 1989. Inhibition by loperamide of mucus secretion in the rat colon *in vivo*. Eur J Pharmacol 170:41-46.

122) Lucas, M. 1982. Restriction of hydrogen and sodium ion diffusion in porcine gastric mucin: a concentration dependent phenomenon. Adv Exp Med Biol 144:193-195.

123) Lucas, M. 1983. Determination of acid surface pH *in vivo* in rat proximal jejunum. Gut 24:734-739.

124) Mack, D. R., W. Neumann, Z. Policova, and P. M. Sherman. 1992. Surface hydrophobicity of the intestinal tract. Am J Physiol 262 (Gastrointest Liver Physiol 25):G171-177.

125) Mack, D. R. and P. M. Sherman. 1991. Mucin isolated from rabbit colon inhibits *in vitro* binding of *Escherichia coli* RDEC-1. Infect Immun 59:1015-1023.

126) Mantle, M. and A. Allen. 1989. Gastrointestinal mucus. In: Gastrointestinal Secretion, Ed J. S. Davison. London: Wright. p. 202-229.

127) Mantle, M. and A. Allen. 1978. A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain. Biochem Soc Trans 6:607-609.

128) Mantle, M. and A. Allen. 1981. Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. Biochem J 195:267-275.

129) Mantle, M., E. Atkins, J. Kelly, E. Thakore, A Buret, and D. G. Gall. 1991. Effects of *Yersinia enterocolitica* infection on rabbit intestinal and colonic goblet cells and mucin: morphometrics, histochemistry, and biochemistry. Gut 32:1131-1138.

130) Mantle, M., L. Basaraba, S. C. Peacock, and D. G. Gall. 1989. Binding of *Yersinia enterocolitica* to rabbit intestinal brush border membranes, mucus, and mucin. Infect Immun 57:3292-3299.

131) Mantle, M., G. G. Forstner, and J. F. Forstner. 1984. Antigenic and structural features of goblet-cell mucin of human small intestine. Biochem J 217:159-167.

132) Mantle, M. and C. Rombough. 1993. Growth in and breakdown of purified rabbit small intestinal mucin by *Yersinia enterocolitica*. Infect Immun 61:4131-4138.

133) Mantle, M. and E. Thakore. 1988. Rabbit intestinal and colonic mucins: isolation, partial characterization, and measurement of secretion using an enzyme-linked immunoassay. Biochem Cell Biol 66:1045-1054.

134) Mantle, M., E. Thakore, R. Mathison, and J. S. Davison. 1991. Intestinal mucin secretion in streptozotocin-diabetic rats: lack of response to cholinergic stimulation and cholera toxin. Dig Dis Sci 36:1574-1581.

135) Masci, E., P. A. Testoni, L. Fanti, M. Guslandi, M. Zuin, and A. Tittobello. 1987. Duodenogastric reflux: correlations among bile acid pattern, mucus secretion, and mucosal damage. Scand J Gastroenterol 22:308-312.

136) Mascolo, N., V. M. Rajendran, and H. J. Binder. 1991. Mechanism of short-chain fatty acid uptake by apical

membrane vesicles of rat distal colon. Gastroenterol 101:331-338.

137) McCormick, B. A., B. A. Stocker, D. C. Laux, and P. S. Cohen. 1988. Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella typhimirium* to colonize the large intestine of streptomycin-treated mice. Infect Immun 56:2209-2217.

138) McIntosh, I. and G. R. Cutting. 1992. Cystic fibrosis transmembrane conductance regulator and the etiology and pathogenesis of cystic fibrosis. FASEB J 6:2775-2782.

139) McKay, D. M., D. W. Halton, M. D. McCaigne, C. F. Johnston, I. Fairweather, and C. Shaw. 1990. *Hymenolepis diminuta*: intestinal goblet cell response to infection in male mice. Exp Parasitol 71:9-20.

140) McSweegan, E., D. H. Burr, and R. I. Walker. 1987. Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT 407 cells. Infect Immun 55:1431-1435.

141) Menguy, R. and A. E. Thompson. 1967. Regulation of secretion of mucus from the gastric antrum. Ann NY Acad Sci 140:797-803.

142) Meshorer, A. 1976. Histological findings in rabbits which died with symptoms of mucoid enteritis. Laboratory Animals 10:199-202.

143) Metcalfe, J. W., K. A. Krogfelt, H. C. Krivan, P. S. Cohen, and D. C. Laux. 1991. Characterization and identification of a porcine small intestine mucus receptor for the K88ab fimbrial adhesin. Infect Immun 59:91-96.

144) Miller, C. O. and J. M. Dunbar. 1933. Change in viscosity of mucin with pH. Proc Soc Exp Biol Med 30:627-629.

145) Miller, H. R. P. 1987. Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. Parasitol 94 Suppl:S77-S100.

146) Miller, H. R. P. and J. F. Huntley. 1982. Protection against nematodes by intestinal mucus. Adv Exp Med Biol 144:243-245.

147) Miller, H. R. P. and Y. Nawa. 1979. *Nippostrongylus brasiliensis*: intestinal goblet-cell response in adoptively immunized rats. Exp Parasitol 47:81-90.

148) Miller, T. A. 1988. Gastroduodenal mucosal defense: Factors responsible for the ability of the stomach and duodenum to resist injury. Surgery 103:389-397.

149) Moore, B. A., K. A. Sharkey, and M. Mantle. 1993. Neural mediation of cholera toxin-induced mucin secretion in the rat small intestine. Am J Physiol 265 (Gastrointest Liver Physiol 28):G1050-G1056.

150) Moqbel, R., D. Wakelin, A. J. MacDonald, S. J. King, R. K. Grencis, and A. B. Kay. 1987. Release of leukotrienes during rapid expulsion of *Trichinella spiralis* from immune rats. Immunol 60:425-430.

151) Moré, J., F. Bénazet, J. Fioramonti, and M.-T. Dray-Lefaix. 1987. Effects of treatment with smectite on gastric acid and intestinal glycoproteins in the rat: a histochemical study. Histochem J 19:665-669.

152) Moré, J., L. Fioramonti, F. Bénazet, and L. Buéno. 1987. Histochemical characterization of glycoproteins present in jejunal and colonic goblet cell of pigs on different diets. Histochemistry 87:189-194.

153) Morton, G. M. and G. W. Stavraky. 1949. A histo-physiological study of the effect of intraarterial injection of acetylcholine upon the gastric mucosa of the dog. Gastroenterol 12:808-820.

154) Mottram, J. C. 1923. Some effects of exposure to radium upon the alimentary canal. Proc R Soc Med 16:41.

155) Mouricout, M. A. and R. A. Julien. 1987. Pilus-mediated binding of bovine enterotoxigenic *Escherichia coli* to calf small intestinal mucins. Infect Immun 55:1216-1223.

156) Murty, V. L. N., J. Piotrowski, A. Czajkowski, A. Slomiany, and B. L. Slomiany. 1993. Inhibition of *Helicobacter pylori* glycosulfatase activity towards human gastric sulfomucin by a gastroprotective agent, sulglycotide. Gen Pharmacol 24:1463-1466.

157) Nadziejko, C. E., B. L. Slomiany, and A. Slomiany. 1993. Most of the lipid in purulent sputum is bound to mucus glycoprotein. Exp Lung Res 19:671-684.

158) Nakamura, A., T. Nakahari, T. Senda, and Y. Imai. 1993. Exocytosis in the lingual mucus cells of *Rana esculenta*

evoked by acetylcholine: observation of living cells by confocal laser scanning microscopy. Jap J Physiol 43:833-846.

159) Negus, V. 1967. The function of mucus: A hypothesis. Proc Roy Soc Med 60:75-77.

160) Neutra, M. R. and J. F. Forstner. 1987. Gastrointestinal mucus--synthesis, secretion, and function. In Physiology of the Gastrointestinal Tract, 2nd ed Ed. L. R. Johnson, New York: Raven Press, p.975-1009.

161) Neutra, M. R., L. J. O'Malley, and R. D. Specian. 1982. Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues. Am J Physiol 242 (Gastrointest Liver Physiol 5):G380-G387.

162) Nevola, J. J., D. C. Laux, and P. S. Cohen. 1987. *In vivo* colonization of the mouse large intestine and *in vitro* penetration of intestinal mucus by an avirulent smooth strain of *Salmonella typhimirium* and its lipopolysaccharide-deficient mutant. Infect Immun 55:2884-2890.

163) Nikkels, R. J., J. W. M. A. Mullink, and J. C. J. Van Vliet. 1976. An outbreak of rabbit enteritis: Pathological and microbiological findings and possible therapeutic regime. Lab Animals 10:195-198.

164) Njoku, O. O. and G. J. Leitch. 1983. Separation of cholera enterotoxin-induced mucus secretion from electrolyte secretion in rabbit ileum by acetazolamide, colchicine, cycloheximide, cytochalasin B and indomethacin. Digestion 27:174-184.

165) Nunn, S., R. St. C. Gilmore, J. A. Dodge, and K. E. Carr. 1990. Exudate variation in the rabbit gastrointestinal tract: a scanning electron microscope study. J Anat 170:87-98.

166) Oderda, G., M. D'Alessandro, P. Mariani, P. Lionetti, M. Bonamico, D. Dell'Olio, and N. Ansaldi. 1993. Prostaglandin E_2 in gastric mucosa of children with *Helicobacter pylori* gastritis: Relation to thickness of mucus gel layer. J Clin Pathol 46:836-839.

167) Oliver, M. G. and R. D. Specian. 1990. Cytoskeleton of intestinal goblet cells: role of actin filaments in baseline secretion. Am J Physiol 259 (Gastrointest Liver Physiol 22):G991-G997.

168) Ollar, R. A. and D. E. S. Stewart-Tull. 1987. Studies on the *Vibrio cholerae* mucinase complex. II. Specific neuraminidase activity measured histochemically in a goblet cell assay. J Med Microbiol 23:227-232.

169) Park, C. M., P. E. Reid, D. A. Owen, J. M. Sanker, and D. A. Applegarth. 1987. Morphological and histochemical changes in intestinal mucosa in the reserpine-treated rat model of cystic fibrosis. Exp Mol Pathol 47:1-12.

170) Pærregaard, A., F. Espersen, O. M. Jenson, and M. Skurnik. 1991. Interactions between *Yersinia enterocolitica* and rabbit ileal mucus: growth, adhesion, penetration, and subsequent changes in surface hydrophobicity and ability to adhere to ileal brush border membrane vesicles. Infect Immun 59:253-260.

171) Peatfield, A. C., P. J. Piper, and P. S. Richardson. 1982. The effect of leukotriene C_4 on mucin release into the cat trachea *in vivo* and *in vitro*. Br J Pharmacol 77:391-393.

172) Pfeiffer, C. J. 1981. Experimental analysis of hydrogen ion diffusion in gastrointestinal mucus glycoprotein. Am J Physiol 240 (Gastrointest Liver Physiol 3):G176-G182.

173) Phillips, T. E., C. Huet, P. R. Bilbo, D. K. Podolsky, D. Louvard, and M. R. Neutra. 1988. Human intestinal goblet cells in monolayer culture: characterization of a mucus secreting subclone derived from the HT29 colon adenocarcinoma cell line. Gastroenterol 94:1390-1403.

174) Phillips, T. E., T. H. Phillips, and M. R. Neutra. 1984. Regulation of intestinal goblet cell secretion. III. Isolated intestinal epithelium. Am J Physiol 247:G674-G681.

175) Phillips, T. E., T. H. Phillips, and M. R. Neutra. 1984. Regulation of intestinal goblet cell secretion. IV. Electrical field stimulation *in vitro*. Am J Physiol 247:G682-G687.

176) Phillips, T. E., T. H. Phillips, and M. R. Neutra. 1989. Cholinergic responsiveness of goblet cells during intestinal maturation. Biol Neonate 55:197-203.

177) Phillips, T. E., W. F. Stenson, and M. R. Neutra. 1989. Lipoxygenase metabolites of arachidonic acid do not induce mucus secretion from rabbit intestinal goblet cells *in vitro*. Prost Leuk Essent Fatty Acids 37:51-55.

178) Phillips, T. E. and J. Wilson. 1993. Morphometric analysis of muous granule depletion and replenishment in rat colon. Dig Dis Sci 38:2299-2304.

179) Pinczower, G. D., R. D. Gianello, R. P. W. Williams, B. N. Preston, H. Preston, and A. W. Linnane. 1993. Monoclonal antibody 4D3 detects small intestinal mucin antigen (SIMA)-glycoprotein in the serum of patients with colorectal cancer. Int J Cancer 54:391-396.

180) Piotrowski, J., A. Czajkowski, F. Yotsumoto, A. Slomiany, and B. L. Slomiany. 1994. Sulglycotide effect on the proteolytic and lpolytic activities of *Helicobacter pylori* toward gastric mucus. Am J Gastroenterol 89:232-236.

181) Quarterman, J. 1987. Metal absorption and the intestinal mucus layer. Digestion 37:1-7.

182) Quigley, E. M. M. and L. A. Turnberg. 1987. pH of the microclimate lining human gastric and duodenal mucosa *in vivo*. Gastroenterol 92:1876-1884.

183) Rabbani, G. H. and W. B. Greenough. 1990. Cholera. In Textbook of Secretory Diarrhea Ed. E. Lebenthal and M. Duffey. New York:Raven Press. p. 233-254.

184) Radwan, K. A., M. G. Oliver, and R. D. Specian. 1990. Cytoarchitectural reorganization of rabbit colonic goblet cells during baseline secretion. Am J Anat 189:365-376.

185) Rechkemmer, G., M. Wahl, W. Kuschinsky, and W. von Engelhardt. 1986. pH-Microclimate at the luminal surface of the intestinal mucosa of guinea pig and rat. Pflügers Arch 407:33-40.

186) Reynolds, I. J., R. J. Gould, and S. H. Snyder. 1984. Loperamide: blockade of calcium channels as a mechanism for antidiarrheal effects. J Pharmacol Exp Ther 231:628-632.

187) Rikihisa, Y., G. C. Johnson, Y.-Z. Wang, S. M. Reed, R. Fertel, and H. J. Cooke. 1992. Loss of absorptive capacity for sodium and chloride in the colon causes diarrhoea in Potomac horse fever. Res Vet Sci 52:353-362.

188) Roberton, M., M. Mantle, R. E. F. Fahim, R. D. Specian, A. Bennick, S. Kawagishi, P. Sherman, and J. F. Forstner. 1989. The putative 'link' glycopeptide associated with mucus glycoproteins. Biochem J 261:637-647.

189) Roomi, N., M. Laburthe, N. Fleming, R. Crowther, and J. Forstner. 1984. Cholera-induced mucin secretion from rat intestine: lack of effect of cAMP, cycloheximide, VIP, and colchicine. Am J Physiol 247:G140-G148.

190) Ross, I. N., H. M. M. Bahari, and L. A. Turnberg. 1982. Studies of the pH gradient across the mucus on rat gastric mucosa *in vivo* and across mucus on human gastric mucosa *in vitro*. Adv Exp Med Biol 144:189-191.

191) Roth, J., P. Greenwell, and W. M. Watkins. 1988. Immunolocalization of blood group A gene specified α1,3N-acetylgalactosaminyl-transferase and blood group A substance in the trans-tubular network of the Golgi apparatus and mucus of intestinal goblet cells. Eur J Cell Biol 46:105-112.

192) Ruseler van Embden, J. G., R. van der Helm, and L. M. van Lieshout. 1989. Degradation of intestinal glycoproteins by *Bacteroides vulgatus*. FEMS Microbiol Lett 49:37-41.

193) Said, H. M., R. Smith, and R. Redha. 1987. Studies on the intestinal surface acid microclimate: developmental aspects. Pediatr Res 22:497-499.

194) Sakata, T. and W. von Engelhardt. 1981. Luminal mucin in the large intestine of mice, rats, and guinea pigs. Cell Tissue Res 219:629-635.

195) Sanchez, R., L. Kanarek, J. Koninkx, H. Hendriks, P. Lintermans, A. Bertels, G. Charlier, and E. Van Driessche. 1993. Inhibition of adhesion of enterotoxigenix *Escherichia coli* cells expressing F17 fimbriae to small intestinal mucus and brush-border membranes of young calves. Microbial Path 15:407-419.

196) Sarosiek, J., B. J. Marshall, D. A. Peura, S. Hoffman, T. Feng, and R. W. McCallum. 1991. Gastroduodenal mucus gel thickness in patients with *Helicobacter pylori*: a method for assessment of biopsy specimens. Am J Gastroenterol 86:729-734.

197) Satchithanandam, S., M. M. Cassidy, A. T. Kharroubi, R. J. Calvert, A. R. Leeds, and G. V. Vahouny. 1990. Alterations in rat intestinal mucin patterns following luminal infusion of acetylsalicylic acid and prostaglandin derivatives. Digest Dis Sci 35:1518-1527.

198) Scheiman, J. M., E. R. Kraus, and C. R. Boland. 1992. Regulation of canine gastric mucin synthesis and phospholipid secretion by acid secretagogues. Gastroenterology 103:1842-1850.

199) Schlichter, L. C. 1982. Unstirred mucus layers: ion exchange properties and effect on ion regulation in *Lymnaea stagnalis*. J Exp Biol 98:363-372.

200) Schrager, J. and M. D. G. Oates. 1974. The isolation and partial characterization of a glycoprotein isolated from human gastric aspirates and from extracts of gastric mucosae. Biochim Biophys Acta 372:183-195.

201) Shea-Donohue, T., E. Danquechin-Dorval, E. Montcalm, H. El-Bayer, A. Durakovic, J. J. Conklin, and A. Dubois. 1985. Alterations in gastric mucus secretion in rhesus monkeys after exposure to ionizing radiation. Gastroenterol 88:685-690.

202) Sherman, P., N. Fleming, J. Forstner, N. Roomi, and G. Forstner. 1987. Bacteria and the mucus blanket in experimental small bowel bacterial overgrowth. Am J Pathol 126:527-534.

203) Shimada, T. 1987. Factors affecting the microclimate pH in rat jejunum. J Physiol 392:113-127.

204) Shimura, S., T. Sasaki, K. Ikeda, H. Sasaki, and T. Takishima. 1988. VIP augments cholinergic-induced glycoconjugate secretion in tracheal submucosal glands. J Appl Physiol 65:2537-2544.

205) Shorrock, C. J. and D. W. Rees. 1988. Overview of gastroduodenal mucosal protection. Am J Med 84(Suppl 2A):25-34.

206) Shorrock, C. J. and D. W. Rees. 1989. Mechanisms of gastric damage by non-steroidal antiinflammatory drugs. Scand J Rheumatol Suppl 78:5-11.

207) Sinkovics, G. 1976. Intestinal flora studies in rabbit mucoid enteritis. Vet Rec 98:151-152.

208) Sinkovics, G. 1978. Rabbit dysentery: 3 Diagnostic differentiation. Vet Rec 103:331-332.

209) Skov Olson, P. 1988. Role of epidermal growth factor in gastroduodenal mucosal protection. J Clin Gastroenterol 10 (Suppl 1):S146-S151.

210) Slomiany, A., K. Okazaki, S. Tamura, and B. L. Slomiany. 1991. Identity of mucin's "118-kDa link protein" with fibronectin fragment. Arch Biochem Biophys 286:383-388.

211) Slomiany, B. L., J. Piotrowski, A. Czajkowski, V. L. N. Murty, and A. Slomiany. 1993. Characterization of gastric mucosal mucin receptor. Biochem Mol Biol Int 31:745-753.

212) Smith, G. W., P. M. Wiggins, S. P. Lee, and C. Tasman-Jones. 1986. Diffusion of butyrate through pig colonic mucus *in vitro*. Clin Sci 90:271-276.

213) Smith, H. 1953. Factors involved in the virulence-enhancing action of mucin. Proc Roy Soc Med 46:787-790.

214) Smith, H., H. T. Zwartow, R. C. Gallop, and P. W. Harris-Smith. 1953. The virulence-enhancing factor of mucins. 7. The remaining components of the "third factor" involved in virulence enhancement. Biochem J 53:673-678.

215) Smithson, K. W., D. B. Millar, L. R. Jacobs, and G. M. Gray. 1981. Intestinal diffusion barrier: Unstirred water layer of membrane surface mucous coat? Science 214:1241-1244.

216) Snyder, J. D. and W. A. Walker. 1987. Structure and function of intestinal mucin: developmental aspects. Int Arch Allergy Appl Immunol 82:351-356.

217) Specian, R. D. and M. R. Neutra. 1980. Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. J Cell Biol 85:626-640.

218) Specian, R. D. and M. R. Neutra. 1982. Regulation of intestinal goblet cell secretion. I. Role of parasympathetic stimulation. Am J Physiol 242 (Gastrointest Liver Physiol 5):G370-G379.

219) Specian, R. D. and M. R. Neutra. 1984. Cytoskeleton of intestinal goblet cells in rabbit and monkey. The theca. Gastroenterol 87:1313-1325.

220) Specian, R. D. and M. G. Oliver. 1991. Functional biology of intestinal goblet cells. Am J Physiol 260 (Cell Physiol 29): C183-C193.

221) Sperber, K., S. Ogata, C. Sylvester, J. Aisenberg, A. Chen, L Mayer, and S. Itzkowitz. 1993. A novel human macrophage-derived intestinal mucin secretagogue: implications for the pathogenesis of inflammatory bowel disease. Gastroenterology 104:1302-1309.

222) Spira, W. M., R. B. Sack, and J. L. Froehlich. 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. Infect Immun 32:739-747.

223) Strocchi, A. and M. D. Levitt. 1991. A reappraisal of the magnitude and implications of the intestinal unstirred layer. Gastroenterol 101:843-847.

224) Strömqvist, M. and H. Gruffman. 1992. Periodic acid/Schiff staining of glycoproteins immobilized on a blotting matrix. Biotechniques 13:744-749.

225) Szentkuti, L., H. Riedesel, M.-L. Enss, K. Gaertner, and W. von Engelhardt. 1990. Pre-epithelial mucus layer in the colon of conventional and germ-free rats. Histochem J 22:491-497.

226) Taatjes, D. J. and J. Roth. 1988. Alteration in sialyltransferase and sialic acid expression accompanies cell differentiation in rat intestine. Eur J Cell Biol 46:289-298.

227) Taatjes, D. J. and J. Roth. 1990. Selective loss of sialic acid from rat small intestinal epithelial cells during postnatal development: demonstration with lectin-gold techniques. Eur J Cell Biol 53:255-266.

228) Taatjes, D. J., J. Roth, J. Weinstein, and J. C. Paulson. 1988. Post-golgi apparatus localization and regional expression of rat intestinal sialyltransferase detected by immunoelectron microscopy with polypeptide epitope-purified antibody. J Biol Chem 263:6302-6309.

229) Targowski, S. and F. Toofanian. 1982. Induction of mucoid enteritis in rabbits by ligation of the cecum or colon. J Am Vet Med Assoc 181:1378-1380.

230) Terakawa, S. and Y. Suzuki. 1991. Exocytosis in colonic goblet cells visualized by video-enhanced light microscopy. Biochem Biophys Res Commun 176:466-472.

231) Thiru, S., G. Devereux, and A. King. 1990. Abnormal fucosylation of ileal mucus in cystic fibrosis: I A histochemical study using peroxidase labelled lectins. J Clin Pathol 43:1014-1018.

232) Timmons, P. M., C.-T. J. Chan, P. W. J. Rigby, and F. Poirier. 1993. The gene encoding the calcium binding protein calcyclin is expressed at sites of exocytosis in the mouse. J Cell Sci 104:187-196.

233) Toofanian, F. 1985. Intestinal disaccharidase and alkaline phosphatase activities in experimental rabbit mucoid enteropathy. Lab Animal Sci 35:624-626.

234) Toofanian, F. and D. W. Hamar. 1986. Cecal short-chain fatty acids in experimental rabbit mucoid enteropathy. Am J Vet Res 47:2423-2425.

235) Toofanian, F. and S. Targowski. 1983. Experimental production of rabbit mucoid enteritis. Am J Vet Res 44:705-708.

236) Toofanian, F. and S. Targowski. 1986. Stimulation of colonic goblet cells by cecal filtrates from rabbits with experimental mucoid enteropathy. Lab Anim Sci 36:157-160.

237) Tzouvelekis, L. S., A. F. Mentis, A. M. Makris, C. Spiliadis, C. Blackwell, and D. M. Weir. 1991. *In vitro* binding of *Helicobacter pylori* to human gastric mucin. Infect Immun 59:4252-4254.

238) van Kruiningen, H. J. and C. B. Williams. 1972. Mucoid enteritis of rabbits. Vet Path 9:53-77.

239) Vasconcellos, C. A., P. G. Allen, M. E. Wohl, J. M. Drazen, P. A. Janmey, and T. P. Stossel. 1994. Reduction in viscosity of cystic fibrosis sputum *in vitro* by gelsolin. Science 263:969-971.

240) Vecchi, M., S. Sakamaki, B. Diamond, A. B. Novikoff, P. M. Novikoff, and K. M. Das. 1987. Development of a monoclonal antibody specifically reactive to gastrointestinal goblet cells. Proc Natl Acad Sci USA 84:3425-3429.

241) Vecchi, M., G. Torgano, M. Monti, E. Berti, D. Agape, M Primignani, G. Ronchi, and R. de Franchis. 1987. Evaluation of structural and secretory glycoconjugates in normal human jejunum by means of lectin histochemistry. Histochem 86:359-364.

242) Verdugo, P. 1990. Goblet cells secretion and mucogenesis. Ann Rev Physiol 52:157-176.

243) Wadolkowski, E. A., D. C. Laux, and P. S. Cohen. 1988. Colonization of the streptomycin-treated mouse large intestine by a human fecal *Escherichia coli* strain: role of adhesion to mucosal receptors. Infect Immun 56:1036-1043.

244) Wadolkowski, E. A., D. C. Laux, and P. S. Cohen. 1988. Colonization of the streptomycin-treated mouse large intestine by a human fecal *Escherichia coli* strain: role of growth in mucus. Infect Immun 56:1030-1035.

245) Walker, R. I. and R. L. Owen. 1990. Intestinal barriers to bacteria and their toxins. Ann Rev Med 41:393-400.

246) Walker, W. A., M. Wu, and K. J. Bloch. 1977. Stimulation by immune complexes of mucus release from goblet cells of the rat small intestine. Science 197:370-371.

247) Wang, Y.-Z., H. J. Cooke, H.-C. Su, and R. Fertel. 1990. Histamine augments colonic secretion in guinea pig distal colon. Am J Physiol 258 (Gastrointest Liver Physiol 21):G432-G439.

248) Wanke, C. A., S. Cronan, C. Goss, K. Chadee, and R. L. Guerrant. 1990. Characterization of binding of *Escherichia coli* strains which are enteropathogens to small-bowel mucin. Infect Immun 58:794-800.

249) Wells, P. D. 1963. Mucin-secreting cells in rats infected with *Nippostrongylus brasiliensis*. Exp Parasitol 14:15-22.

250) Westergaard, H. and J. M. Dietschy. 1974. Delineation of the dimensions and permeability characteristics of the two major diffusion barriers to passive mucosal uptake in the rabbit intestine. J Clin Invest 54:718-732.

251) Whitney, J. C. 1976. A review of non-specific enteritis in the rabbit. Laboratory Animals 10:209-221.

252) Wien, E. M. and D. R. Van Campen. 1991. Mucus and iron absorption regulation in rats fed various levels of dietary iron. J Nutr 121:92-100.

253) Williams, S. E. and L. A. Turnberg. 1980. Retardation of acid diffusion by pig gastric mucus: a potential role in mucosal protection. Gastroenterol 79:299-304.

254) Williams, S. E. and L. A. Turnberg. 1981. Demonstration of a pH gradient across mucus adherent to rabbit gastric mucosa: evidence for a "mucus-bicarbonate" barrier. Gut 22:94-96.

255) Winet, H. 1976. Ciliary propulsion of objects in tubes: wall drag on swimming *Tetrahymena* (Ciliata) in the presence of mucin and other long chain polymers. J Exp Biol 64:283-302.

256) Xu, G., L. Huan, I. Khatri, U. S. Sajjan, D. McCool, D. Wang, C. Jones, G. Forstner, and J. Forstner. 1992. Human intestinal mucin-like protein (MLP) is homologous with rat MLP in the C-terminal region, and is encoded by

a gene on chromosome 11 p 15.5. Biochem Biophys Res Comm 183:821-828.

257) Xu, G., L.-J. Huan, I. A. Khatri, D. Wang, A. Bennick, R. E. F. Fahim, G. G. Forstner, and J. F. Forstner. 1992. cDNA for the carboxyl-terminal region of a rat intestinal mucin-like peptide. J Biol Chem 267:5401-5407.

258) Yagi, T., Y. Miyawaki, A. Nishikawa, S. Horiyama, K. Yamauchi, and S. Kuwano. 1990. Prostaglandin E_2-mediated stimulation of mucus synthesis and secretion by rhein anthrone, the active metabolite of sennosides A and B, in the mouse colon. J Pharm Pharmacol 42:542-545.

259) Yamamoto, T., Y. Koyama, M. Matsumoto, E. Sonoda, S. Nakayama, M. Uchimura, W. Paveenkittiporn, K. Tamura, T. Yokota, and P. Echeverria. 1992. Localized, aggregative, and diffuse adherence to HeLa cells, plastic, and human small intestines by *Escherichia coli* isolated from patients with diarrhea. J Infect Dis 166:1295-1310.

260) Yamamoto, T. and T. Yokota. 1988. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. Infect Immun 56:2753-2759.

261) Yamamoto, T. and T. Yokota. 1988. *Vibrio cholerae* non-O1: production of cell-associated hemagglutinins and *in vitro* adherence to mucus coat and epithelial surfaces of the villi and lymphoid follicles of human small intestines treated with formalin. J Clin Microbiol 26:2018-2024.

262) Yolken, R. H., C. Ojeh, I. A. Khatri, U. Sajjan, and J. F. Forstner. 1994. Intestinal mucins inhibit rotavirus replication in an oligosaccharide-dependent manner. J Infect Dis 169:1002-1006.

263) Zahm, J. M., D. Pierrot, C. Fuchey, J. Levrier, D. Duval, K. G. Lloyd, and E. Puchelle. 1989. Comparative rheological profile of rat gastric and duodenal gel mucus. Biorheology 26:813-822.

264) Zaus, E. A. and L. S. Foskick. 1934. The antipeptic influence of gastric mucin. Am J Digest Dis 1:177-178.

265) Zenian, A. J. and F. D. Gillin. 1987. Intestinal mucus protects *Giardia lamblia* from killing by human milk. J Protozool 34:22-26.

266) Zijlstra, F. J., E. D. Srivastava, M. Rhodes, A. P. M. van Dijk, F. Fogg, H. J. Samson, M. Copeman, M. A. H.

Russell, C. Feyerabend, G. T. Williams, R. D. Pullan, G. A. O. Thomas, M. Van Blankenstein, J. H. P. Wilson, A. Allen, and J. Rhodes. 1994. Effect of nicotine on rectal mucus and mucosal eicosanoids. Gut 35:247-251.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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